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**Associations of gene polymorphisms and
nutrition with calcium homeostasis and
bone mineral density**

- Studies on skeletal nutrigenetics

ACADEMIC DISSERTATION

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Nothing in this world is to be feared

...only understood.

Marie Curie

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TIIVISTELMÄ, Finnish summary

Perimä selittää suuren osan kalsiumin aineenvaihdunnassa ja luun vahvuudessa esiintyvistä vaihtelusta, ja useat geenit säätelevät alttiutta sairastua osteoporoosiin. Luun vahvuus muotoutuu siis perimän ja elintapojen yhteisvaikutuksena. Monilla ravintotekijöillä on merkitystä luun hyvinvoinnille koko elinkaaren ajan. Ravinnon ja perimän yhteisvaikutuksia selvittävä nutrigenetiikka on uusi ja merkittävä tutkimusalue osteoporoosin etiologiassa. Vaikka osteoporoosin perinnöllisyyden selvittämisessä on viime vuosina saavutettu merkittäviä edistysaskelia, toistaiseksi tutkimustulokset ovat melko ristiriitaisia.

Väitöskirjatyössä tutkittiin laktaasientsyymiä, D-vitamiinireseptoria, kalsiumia sitovaa reseptoria sekä lisäkilpirauhashormonia (PTH) koodaavien geenien normaalivaihtelun eli geenipolymorfismien yhteyksiä ja yhteisvaikutuksia luuston terveyteen eri ikäisillä suomalaisilla. Kyseiset kandidaattigeenit valittiin tutkimukseen, koska niiden merkitys luukudoksen biologiassa tunnetaan ja niiden polymorfiavaihtelun (=genotyyppien) esiintyvyys geneettisesti homogeenisessa suomalaisväestössä on riittävä kalsiumin aineenvaihdunnassa ja luun vahvuudessa esiintyvien erojen selvittämiseksi. Poikkileikkaustutkimusten kohderyhminä olivat 14–16-vuotiaat tytöt ja pojat, 31–43-vuotiaat naiset ja miehet sekä ryhmä 22–45- ja 48–65-vuotiaita naisia. Seuranta-tutkimukseen osallistuneet olivat tutkimuksen alkaessa 3–18-vuotiaita ja viimeisimmän seurannan aikana 32–41-vuotiaita. Veri- ja virtsanäytteistä tutkittiin kalsiumin ja luun aineenvaihduntaa kuvaavia merkkiaineita. Luun vahvuutta mitattiin DXA-menetelmällä käsivarresta, lannerangasta ja reisiluun kaulaosasta sekä ultraääneen perustuvalla menetelmällä kantaluusta. Luustoon vaikuttavat elintavat kartoitettiin huolellisesti, jolloin voitiin selvittää luotettavasti perimän osuutta sekä ravinnon ja geenien yhteisvaikutuksia kalsiumin aineenvaihduntaan ja luun vahvuuteen.

Perinnöllinen laktoosi-intoleranssi (C/C₋₁₃₉₁₀-genotyyppi) oli yhteydessä vähäisempään maidon käyttöön lapsuudesta alkaen altistaen erityisesti naiset riittämättömälle kalsiumin saannille. Vähälaktoosisten maitojen ja maitovalmisteiden käytön havaittiin suojaavan C/C₋₁₃₉₁₀-genotyyppiä suosituksia niukemmalta kalsiumin saannilta nuorella aikuisiällä. Luun huippumassan saavuttamisen jälkeen toteutetussa 12 vuoden seurannassa havaittiin luun mineraalitiheyden pienenevän erityisesti reisiluun kaulaosassa. Luumassan menetys oli miehillä naisia yleisempää. Perimältään C/C₋₁₃₉₁₀-genotyyppiä olevat miehet saattavat olla alttiimpia luumassan menetykselle nuorella aikuisiällä, joskin kalsiumin saanti näyttää suomalaisväestössä selittävän luumassan muutoksia paremmin kuin laktaasigenotyyppi.

Kasvavilla nuorilla D-vitamiinireseptorin (VDR) *BsmI*-polymorfian havaittiin olevan yhteydessä käsivarren luumassaan ja *FokI*-polymorfian kantaluusta ultraäänellä mitattuun luun vahvuuteen. Luun mineraalimäärä käsivarressa oli pienin *Bb*-genotyypillä ja ultraäänen vaimeneminen (BUA) kantaluussa suurin *Ff*-genotyypillä. Aikuisilla *FokI*-polymorfian ei todettu selittävän eroja kalsiumin aineenvaihdunnassa tai luun mineraalitiheydessä. Nuorilla naisilla ja vaihdevuosi-ikä ohittaneilla naisilla *BsmI*-polymorfia ei ollut yhteydessä kalsiumin imeytymiseen tai luun aineenvaihdunnan merkkiaineisiin, mutta *BB*-genotyypillä D-vitamiinitilaa kuvaava seerumin 25OHD-pitoisuus ja lannerangan luuntiheys olivat suurimmat muihin genotyypeihin verrattuna. Tulosten tulkinnassa on otettava huomioon, että tutkimusaineistojen koko ei riitä yleistettävien johtopäätösten tekemiseen.

Kalsiumia sitovan reseptorin (CaSR) 986S-alleeli oli yhteydessä suurempaan seerumin ionisoituneen kalsiumin pitoisuuteen nuorilla aikuisilla kuin 986A-alleeli, mikä vahvistaa aiempia muista väestöistä saatuja tutkimustuloksia. Toisaalta A986S-polymorfialla ei havaittu yhteyksiä muihin kalsiumin aineenvaihduntaa tai luun vahvuutta kuvaaviin muuttujiin. Lisäkilpirauhashormonin *BstBI*-geenipolymorfian *bb*-genotyyppi oli yhteydessä pienempään käsivarren luun mineraalitiheyteen ja ultraäänellä mitattuun kantaluun vahvuuteen. *BstBI*-geenipolymorfian vaikutusmekanismi luun vahvuuteen jäi selvittämättä, sillä kalsiumin ja luun aineenvaihdunnan merkki-aineissa ei havaittu genotyyppien välisiä eroja.

Nuorilla naisilla D-vitamiinireseptorin *FokI*-polymorfialla ja suolan saannilla todettiin yhteisvaikutus kalsiumin eritykseen. Suolan saannin lisääntyminen lisäsi *F*-alleelilla kalsiumin eritystä. Nuorilla aikuisilla havaittu *FokI*- and *BstBI*-polymorfioiden yhteisvaikutus seerumin PTH-pitoisuuteen ja kalsiumin eritykseen on aiemmin julkaisematon tutkimushavainto.

Yhteenvedona tutkimuksista voidaan todeta, että suomalaisväestössä vähälaktoosisten maitojen ja maitovalmisteiden käyttö näyttää suojaavan geneettisestä laktoosin imeytymishäiriöstä kärsiviä niukemmalta kalsiumin saannilta ja luuston kunnan heikkenemiseltä. D-vitamiinireseptorin, kalsiumia sitovan reseptorin ja parathormonin geenipolymorfioilla havaittiin yhteyksiä kalsiumin aineenvaihduntaan ja luun vahvuuteen eri ikäisillä suomalaisilla, mutta yleistettävien johtopäätösten tekemiseksi tarvitaan jatkotutkimuksia näiden perinnöllisten tekijöiden vaikutusmekanismeista sekä ravinnon ja geenien yhteisvaikutusten merkityksestä luuston terveydelle. Lisätutkimukset tulisi tehdä suuremmilla väestöotoksilla, jotka edustavat luuston elinkaaren eri vaiheita, ja luun vahvuutta tulisi mitata monipuolisemmilla mittausten menetelmillä kuten perifeerisellä tietokonetomografialla (pQCT).

Laaksonen Marika. Associations of gene polymorphisms and nutrition with calcium homeostasis and bone mineral density - Studies on skeletal nutrigenetics (dissertation). Helsinki, University of Helsinki. 2008.

ABSTRACT

Heredity explains a major part of the variation in calcium homeostasis and bone strength, and the susceptibility to osteoporosis is polygenetically regulated. Bone phenotype results from the interplay between lifestyle and genes, and several nutritional factors modulate bone health throughout life. Thus, nutrigenetics examining the genetic variation in nutrient intake and homeostatic control is an important research area in the etiology of osteoporosis. Despite continuing progress in the search for candidate genes for osteoporosis, the results thus far have been inconclusive.

The main objective of this thesis was to investigate the associations of lactase, vitamin D receptor (VDR), calcium sensing receptor (CaSR) and parathyroid hormone (PTH) gene polymorphisms and their interactions with bone health in Finns at varying stages of the skeletal life span. This thesis focuses on the genes that have a known role in bone biology and measurable biomarkers or intermediate endpoints in calcium homeostasis and bone mineral density (BMD), and on those polymorphisms in these genes that have a sufficient prevalence in the Finns. The subjects in cross-sectional studies were 14-16-year-old girls and boys, 31-43-year-old women and men (FINRISK survey), and a group of 22-45- and 48-65-year-old women. The subjects of the Young Finns Cohort were 3-18 years old at baseline and 32-41 years old in the follow-up. Markers of calcium homeostasis and bone remodelling were measured from blood and urine samples. BMD at distal forearm, lumbar spine and femoral neck was measured with dual energy x-ray absorptiometry (DXA) method and at calcaneus with quantitative ultrasound method. Lifestyle factors were assessed with questionnaires in order to examine the relationships of diet-gene interactions with calcium homeostasis and BMD.

Genetic lactase non-persistence (the C/C₋₁₃₉₁₀ genotype) was associated with lower consumption of milk from childhood, predisposing females in particular to inadequate calcium intake. In young adults with the C/C₋₁₃₉₁₀ genotype, consumption of low-lactose milk and milk products was shown to decrease the risk for inadequate calcium intake. In 12-year follow-up in young adulthood, bone loss was more common in males than in females and seemed to occur soon after peak bone mass attainment mainly at the femoral neck. Young males with the lactase C/C₋₁₃₉₁₀ genotype may be more susceptible to bone loss than males with the other two genotypes; however, calcium intake predicts changes in bone mass more than the lactase genotype.

In adolescents, the *BsmI* polymorphism of the VDR gene was associated with forearm bone mass (BMC), and *FokI* polymorphism with calcaneal ultrasound values. BMC at the distal forearm was lowest for the *Bb* genotype and calcaneal ultrasound attenuation (BUA) highest for the *Ff* genotype. In adults, the *FokI* polymorphism was not related to the determinants of calcium and bone metabolism nor to the peripheral BMD. In pre- and postmenopausal women, the *BsmI* polymorphism was not related to intestinal calcium absorption or markers of bone remodelling, but the *BB* genotype was associated with higher serum hydroxyvitamin D (S-25OHD) and showed a trend toward higher lumbar spine BMD than other genotypes. However, the study populations were too small to make strong conclusions about the role of VDR polymorphisms in the regulation of calcium and bone metabolism and bone mineral density.

In young adults, the 986S allele of the CaSR gene was associated with higher than average serum ionized calcium concentrations, which confirms the results from some previous studies in other populations. However, CaSR A986S genotypes were not associated with other markers of calcium homeostasis or with forearm BMD and calcaneal ultrasound values. The *bb* genotype of the PTH *BstBI* polymorphism was related to the lowest forearm BMD and calcaneal BUA values, but because no differences were found in the markers of calcium and bone metabolism, the mechanism by which this polymorphism regulates BMD requires further study.

In young adulthood, the *FokI* polymorphism and sodium intake showed an interaction effect on urinary calcium excretion. In females, the *F* allele was associated with increased calcium excretion due to increasing sodium excretion. A novel gene-gene interaction between the VDR *FokI* and PTH *BstBI* gene polymorphisms was found in the regulation of PTH secretion and urinary calcium excretion.

In conclusion, this study suggests that in Finnish population, a preference for low-lactose milk and milk products may protect those with the genetic lactase non-persistence (C/C₁₃₉₁₀ genotype) from inadequate calcium intake and deterioration of bone health. Furthermore, this thesis showed associations of the VDR *FokI*, the CaSR A986S and the PTH *BstBI* polymorphisms with calcium homeostasis and BMD. However, further research should be carried out with more number of subjects at varying stages of the skeletal life span and more detailed measurements of bone strength such as peripheral quantitative computed tomography (pQCT). Further research should concern mechanisms by which genetic variants affect calcium homeostasis and bone strength, and the role of diet-gene and gene-gene interactions in the pathogenesis of osteoporosis.

ABBREVIATIONS

1,25(OH) ₂ D	1,25-dihydroxyvitamin D, calcitriol
25OHD	25-hydroxyvitamin D
95%CI	95% confidence intervals
AHSG	alpha2-HS-glycoprotein
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ApoE	apolipoprotein E
ATP	adenosine triphosphate
BALP	bone-specific alkaline phosphatase
BGP	osteocalcin
BMC	bone mineral content
BMD	bone mineral density
BMU	basic multicellular unit
BUA	broadband ultrasound attenuation
CaSR	calcium sensing receptor
Cdx-2	intestine-specific homeodomain-containing transcription factor
COLIA1	collagen type 1 α
CPBA	competitive protein binding assay
CV	coefficient of variation
DNA	deoxyribonucleic acid
DXA	dual energy x-ray absorptiometry
ESR1	estrogen receptor 1 gene
FFQ	food frequency questionnaire
GH1	growth hormone 1
HWE	Hardy-Weinberg equilibrium
ICTP	cross-linked telopeptide of type I collagen
IGF-1	insulin-like growth factor 1
IL-6	interleukin 6
IL-10	interleukin 10
IRMA	immunoradiometric assay
LCT	lactase-phlorizin hydrolase
LD	linkage disequilibrium
LOD	linkage of disease
LRP5	low density lipoprotein receptor-related protein 5
LSC	least significant change
LSD	Fisher's least significant difference <i>post hoc</i> test
MRI	magnetic resonance imaging

mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase
OPG	osteoprotegerin
PCR	polymerase chain reaction
PICP	carboxyterminal propeptide of collagen type I
PTH	parathyroid hormone
PTHr1	parathyroid hormone type I receptor
RANOVA	repeated measures analysis of variance
PBM	peak bone mass
pQCT	peripheral quantitative computed tomography
QUS	quantitative ultrasound
RANKL	receptor activator of nuclear factor- κ B ligand
RIA	radioimmunoassay
S-25OHD	serum 25-hydroxy-vitamin-D concentration
SD	standard deviation
SEM	standard error of mean
S-iCa	serum ionized calcium concentration
S-iPTH	serum intact parathyroid hormone
SNP	single nucleotide polymorphism
SOS	speed of ultrasound
SOST	sclerostin
S-P	serum phosphorous concentration
TGF- β 1	transforming growth factor β 1
TNFR2	tumour necrosis factor receptor 2
U-Ca	urinary calcium excretion
U-Na	urinary sodium excretion
UTR	untranslated region
VDR	vitamin D receptor
χ^2	Pearson's chi-square
ZBTB40	zinc finger and BTB domain containing 40 gene

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred in the text by Roman numerals (I-IV).

- I **Laaksonen MML**, Impivaara O, Viikari JSA, Sievänen H, Lehtimäki TJ, Lamberg-Allardt CJE, Kärkkäinen MUM, Välimäki M, Heikkinen J, Kröger LM, Kröger HPJ, Jurvelin JS, Kähönen MAP, Raitakari OT, and the Cardiovascular Risk in Young Finns Study Group. Associations of genetic lactase non-persistence and sex with bone loss in young adulthood. Submitted for publication in Bone.
- II **Laaksonen M**, Kärkkäinen M, Outila T, Rita H, Lamberg-Allardt C. Vitamin D receptor gene start codon polymorphism (*FokI*) is associated with forearm bone mineral density and calcaneal ultrasound in Finnish adolescent boys but not in girls. J Bone Miner Metab 2004;22:479-485.
- III **Laaksonen M**, Kärkkäinen M, Outila T, Vanninen T, Ray C, Lamberg-Allardt C. Vitamin D receptor gene *BsmI* polymorphism in Finnish premenopausal and postmenopausal women: its association with bone mineral density, markers of bone turnover and intestinal calcium absorption, with adjustment for lifestyle factors. J Bone Miner Metab 2002;20:383-390.
- IV **Laaksonen MML**, Outila TA, Kärkkäinen MUM, Kemi VE, Rita HJ, Valsta L, Perola M, Lamberg-Allardt CJE. The associations of vitamin D receptor, calcium-sensing receptor and parathyroid hormone gene polymorphisms with calcium homeostasis and peripheral bone density in adult Finns. In revision for publication in Journal of Nutrigenetics and Nutrigenomics.

In addition, unpublished results are presented.

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Contribution of authors to papers I-IV

- I The author planned the study with the co-authors. The author recruited the subjects, organised the bone density measurements and recorded the data. The author carried out the statistical analysis. The author wrote the manuscript and the co-authors participated by giving comments and suggestions.

- II The author planned the study with the co-authors. The author was responsible for the isolation of DNA and genotyping of the subjects. The author carried out the statistical analysis. The author wrote the manuscript and the co-authors participated by giving comments and suggestions.

- III The author planned the study with the co-authors. The author was responsible for the isolation of DNA and genotyping of the subjects. The author carried out the statistical analysis. The author wrote the manuscript and the co-authors participated by giving comments and suggestions.

- IV The author planned the study with the co-authors. The author was responsible for the genotyping of the subjects. The author carried out the statistical analysis. The author wrote the manuscript and the co-authors participated by giving comments and suggestions.

1 INTRODUCTION

In the early 1900s, Archibald Garrod suggested that “the influences of diet and diseases might mask some of the inborn errors of metabolism” (Hunter 2005). Since the days of Garrod, it has been recognized that the etiology of complex diseases involves the interplay between environmental and genetic factors: susceptibility genes modify the effects of environmental risk factors that either exacerbate or suppress disease progression. Genetic epidemiology is concerned with understanding the heritable aspects of disease risks and their associations with environmental factors, and ultimately to contribute to a molecular understanding of disease pathogenesis.

Recently, the completion of the Human Genome Project expanded our ability to define genetic differences at the DNA sequence level. This genomic revolution has introduced nutritional science to new methods and technical applications that provide tools for developing a more holistic concept of gene-diet-disease interactions. Nutrigenetics aims to gain a better understanding of how individual genetic make-up contributes to health and disease susceptibility due to inherited differences in factors determining food choices, as well as in factors determining bioavailability and metabolism of nutrients.

Osteoporosis is sometimes called “a silent disease” because it often develops slowly and seldom gives any warning signs until the bone is so fragile that it is highly susceptible to breakage. A maternal history of osteoporosis or fractures, female gender, advanced age, disturbances in hormone or calcium metabolism, smoking, low calcium intake and poor vitamin D status, physical inactivity, and diseases and medications that affect calcium and bone metabolism are well-known risk factors for low bone mineral density (BMD), osteoporosis and fractures. The evidence is convincing that peak bone mass (PBM), the highest amount of bone mass attained in young adulthood, is a strong predictor for osteoporosis later in life. From this viewpoint, the emphasis is on prevention of osteoporosis and development strategies for early identification of individuals at high risk. Heredity has a major role in regulation of calcium homeostasis and bone strength; thus, the genetics of osteoporosis has been extensively studied in recent years. Although there has been progress towards identifying candidate genes for osteoporosis, so far the results have been inconsistent, and the contribution of genetic variants to regulating bone mass and susceptibility to osteoporosis remain unresolved.

This thesis investigates skeletal nutrigenetics in Finns, focusing on polymorphisms in genes that are involved in the regulation of calcium homeostasis and bone strength. The studies in this thesis reflect advances during the last decade in research on the inheritance factors for calcium homeostasis and bone strength. The first studies concern the vitamin D

receptor gene, which is currently considered to be the first-discovered candidate gene for osteoporosis. The most recent studies reported in this thesis investigate a more novel candidate gene, namely the gene encoding the intestinal lactase enzyme. This thesis adds a few pieces of information to the unfinished and diverse puzzle of skeletal nutrigenetics.

2 REVIEW OF THE LITERATURE

2.1 Genetics of complex diseases

2.1.1 Strategies to study the genetic background of complex diseases

In genetic epidemiology, the main strategies for studying the genetic factors for complex diseases are genome-wide linkage and association studies, and candidate gene approaches (Table 1)(Ralston and de Crombrughe 2006). Genetic linkage analyses in experimental animal models of osteoporosis typically use mouse strains with high or low BMD. Linkage analyses in families dissect the inheritance of the disease or the defined phenotype in relation to the cosegregation of genetic markers within a pedigree (Lander and Schork 1994, Hobson and Ralston 2001). Allele-sharing methods in sib-pairs involve testing whether the affected relatives inherit the same specific genetic region more often than expected based on random Mendelian segregation (Lander and Schork 1994). Linkage between the marker and the inherited phenotype is evaluated by a LOD-score, meaning the logarithm of the odds that the locus is linked with marker rather than unlinked (Stewart and Ralston 2000). The candidate gene method with a case-control approach involves comparisons of the frequencies of candidate gene alleles in affected and unaffected individuals. Association studies at the population level have been the main method for studying human genetics of multifactorial and polygenetic diseases, and their focus has been on single nucleotide polymorphisms (SNPs) of a particular candidate gene. Recently, a genome-wide approach has been adopted in association studies, since scanning for many SNPs distributed across several different genes has become possible without making any assumptions about the identity of the genes.

Table 1. Different strategies to study genetic factors of complex diseases (modified from Ralston and de Crombrughe 2006, Dvornyk et al. 2003).

Genetic approach	Strengths	Weaknesses
Genome-wide linkage studies in animal models	<ul style="list-style-type: none"> - Large progeny - Genetic homogeneity - Confounding from environment controlled 	<ul style="list-style-type: none"> - Risk of discordant regulatory genes
Genome-wide linkage studies in humans - families and sib-pairs	<ul style="list-style-type: none"> - Successful in identifying rare monogenic diseases 	<ul style="list-style-type: none"> - Low statistical power to detect genes with modest effects - Limited number of suitable families or sib-pairs - Limited generalizability at population level
Candidate gene association in humans - case-control studies - population-based	<ul style="list-style-type: none"> - Easy to carry out - Small effects can be detected - Evaluation in a population context 	<ul style="list-style-type: none"> - Linkage disequilibrium with causal variants in other genes - Confounding from environment difficult to control - Small sample sizes - Population stratification
Genome-wide association in humans - population based	<ul style="list-style-type: none"> - Successful in identifying genetic variants of complex diseases 	<ul style="list-style-type: none"> - Differences in enrolment criteria and study designs - Genetic heterogeneity between populations

Single nucleotide polymorphisms

Single nucleotide polymorphisms are the most abundant form of DNA variation in the human genome. On average, every kilobase has a SNP that can be used as a marker on the human genome, and there are over 10 million SNPs deposited in the SNP database (www.ncbi.nlm.nih.gov/SNP) (see Lee 2007). For a polymorphism to be a SNP, incidence of genetic variant must be at least 1% of the population, although a common polymorphic variant that occurs in 10-50% of the population is more relevant from a public health perspective. An important characteristic of SNPs is that they are thought to have very low mutation rates in humans (Wang et al. 1998). The majority of SNPs do not appear to cause a disease, however, they may assist in determining the likelihood that a particular abnormality may occur or they may be linked to an increased disease risk (see Duff 2006). The genetic component of a complex disease is the combination of several SNPs that each may have a relatively small but clinically important contribution to pathogenesis of the disease. SNPs that are physically close together on a chromosome can be in linkage disequilibrium (LD), which means that they are inherited together more frequently than predicted under Mendel's law of independent assortment. Thus, instead of identification of a single causal SNP, haplotype analysis has the advantage of locating susceptibility regions with disease-associated variants (Haiman et al. 2003).

A major challenge in assessing candidate genes for complex diseases is that the SNPs chosen should have a relevant role in the etiology of the disease (Rebbeck et al. 2004). The knowledge of the gene and SNP functions is crucial for the study design and interpretation of the results, because the relevant SNPs are relatively rare compared with the total number of SNPs in the human genome (Sachidanandam et al. 2001). Regulatory and coding SNPs are of particular interest for molecular-association studies since regulatory SNPs can influence the expression or tissue-specific functions of proteins, while coding SNPs can alter their amino acid composition. For instance, SNPs can affect the kinetic parameters of enzymes, the DNA binding of transcription factors, the function of transmembrane receptors, or the role of structural proteins in tissue architecture. Furthermore, the prevalence of the SNPs examined should be sufficiently high within the population studied. If the prevalence of an allele in the population is very rare, the number of subjects in the study has to be increased to achieve sufficient statistical power. Moreover, the penetrance of a SNP associated with a disease should be sufficiently high to result in a clear aggregation of cases, although the penetrance may be modified by environmental and life-style factors (Narod 2002). Finally, the effects of SNPs should be measurable at the levels of risk factors, intermediate end points or outcomes of the disease.

2.1.2 Interplay between diet and genes

Because long exposure to a particular dietary pattern may be necessary for disease development, diet is probably the most influential environmental factor modulating phenotypes for polygenic diseases. *Nutritional genomics* is the scientific study of how genetic factors and bioactive food components interact, and how health consequences of nutrient intake and eating behaviours vary across individuals (Gillies 2003) (Figure 1).

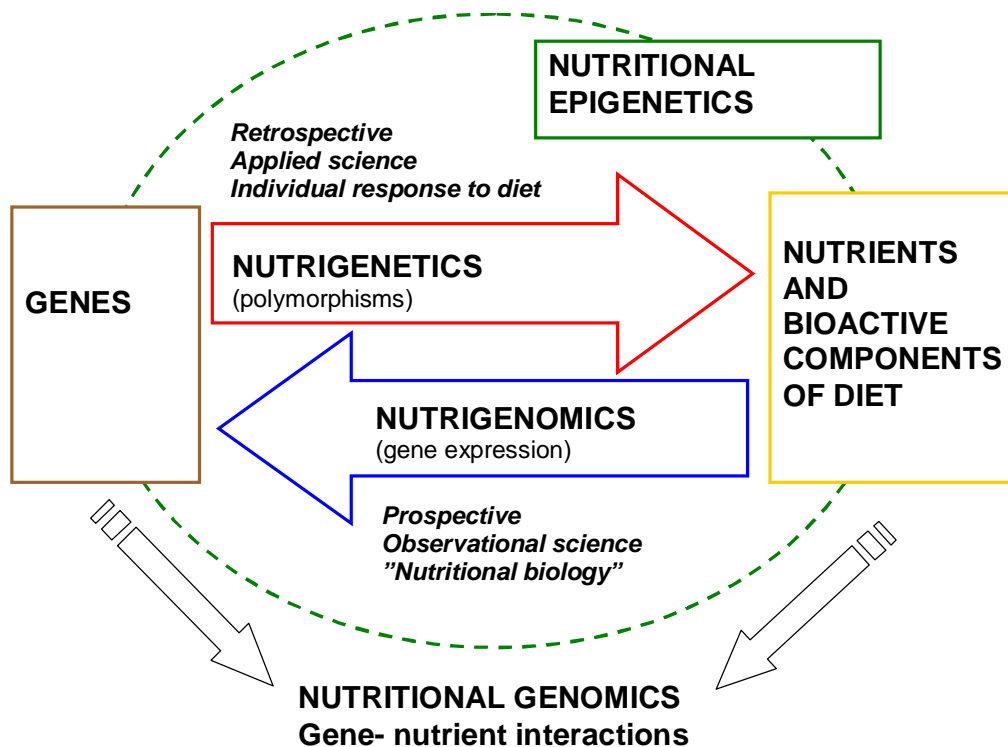


Figure 1 Nutritional genomics studies the interactions between genes and nutrients or bioactive components of diet (modified from Gillies 2003).

Nutrigenetics examines genetic variation in food choices and in metabolic pathways and homeostatic control of nutrients and bioactive food components. Broad evidence suggests that variations in SNPs are associated with differences in the absorption and metabolism of nutrients, thereby contributing to health and disease risks. One of the classic examples is the apolipoprotein E (apoE) polymorphism, which predicts lipid response to changes in dietary fatty acids, and has been linked to risk and risk factors for atherosclerosis (see e.g. Minihane et al. 2007). On the other hand, changes in dietary intake or food supply may hide the detrimental phenotypic effects of a SNP, thereby allowing these variants to be inherited and become established in a population – this phenomenon is referred to as genetic rescue (Stover 2006). Lactase persistence is a good example of a polymorphic human trait for which allele frequencies clearly have been affected by selection due to

improved nutrition. Lactase non-persistence is the ancestral state that dominated until the invention of agriculture around 10 000 years ago created a strong selective advantage to those who can drink milk as adults, since milk improves nutrition, prevents dehydration and provides calcium (Bersaglieri et al. 2004). The populations of Northern Europe and certain nomadic tribes of Africa and Asia have histories of drinking fresh milk rich in lactose, whereas other populations have adapted to milk consumption by fermenting fresh milk to products with lower lactose content such as cheese and yogurt (Enattah et al. 2002, Harvey et al. 1998, Hollox et al 2001).

Increasing evidence suggests that various features in disease etiology are consistent with epigenetic mechanisms resulting from fetal or lifelong environmental influence or stochastic events (see Feil 2006). *Nutritional epigenetics* refers to either permanent or reversible heritable patterns of gene expression that occur without changes in DNA sequences as a result of adaptation to changes in dietary intake or food supply (Jiang et al. 2004). The two main epigenetic mechanisms include DNA methylation and covalent modifications of histones, both of which affect gene expression by altering transcription factor accessibility (Junien 2006, Gallou-Kabani et al. 2007). A recent study showed that, although monozygotic twins are epigenetically indistinguishable during the early years of life, at an older age they have dissimilar epigenetic profiles, and the most disparate profiles were found in older twins and those with a history of non-shared environments (Fraga et al. 2005). Another study in British women indicated that the maternal vitamin D status during pregnancy and placental calcium transfer are significantly correlated with bone mineral accrual in the offspring at 9 years of age, due to fetal programming of endocrine systems that influence skeletal metabolism (Javaid et al. 2006). Furthermore, a Swedish study showed that if food was not readily available during the mother's or father's slow growth period in childhood (8-10 years for girls and 9-12 years for boys), the cardiovascular disease mortality of the offspring was low (Kaati et al 2002).

Nutrigenomics focuses on the effects of essential nutrients and other bioactive food components on the regulation of gene expression. Several bioactive food components such as vitamins, minerals, phytochemicals and macronutrients can act as labile regulators of gene transcription and translation in dose- and time-dependent manners. For example, the active vitamin D metabolite $1,25(\text{OH})_2\text{D}$ regulates calcium and bone metabolism by up- or down regulating genes that code for the key regulatory proteins of bone metabolism such as osteocalcin, osteopontin, calbindin, 24-hydroxylase and parathyroid hormone (PTH)(Nagpal et al. 2005). The development of microarray technology has provided a powerful tool for examining the potential sites of action of food components and a simultaneous assessment of the transcription of tens of thousands of genes. However, the quantity and duration of the exposure are critical factors when evaluating results from a

microarray since this technology only provides a "snap-shot" of the adaptive processes that occur after the ingestion of foods (Trujillo et al. 2006).

2.2 Bone biology and osteoporosis

2.2.1 Calcium homeostasis

Regulation of calcium homeostasis

Calcium serves two major functions for bone: it is a principal component of the mineral portion of bone and serves as an indirect regulator of skeletal remodelling (Heaney and Weaver 2005). In addition to the functions in bone tissue, calcium controls several essential cellular functions, such as neurotransmission, muscle contraction, hormone and enzyme secretion, and blood clotting. The serum ionized-calcium concentration (S-iCa) is the biologically active fraction of the serum calcium, and its concentration in serum varies within very narrow limits (1.15-1.35 mmol/l). Calcium homeostasis is tightly regulated by the actions of PTH, $1,25(\text{OH})_2\text{D}$ and calcitonin in the kidney, bone and intestine (Figure 2). Because the three tissues supporting S-iCa levels partly operate independently of one another, altered calcium metabolism in any of them can cause deterioration of bone health (Weaver and Heaney 2006).

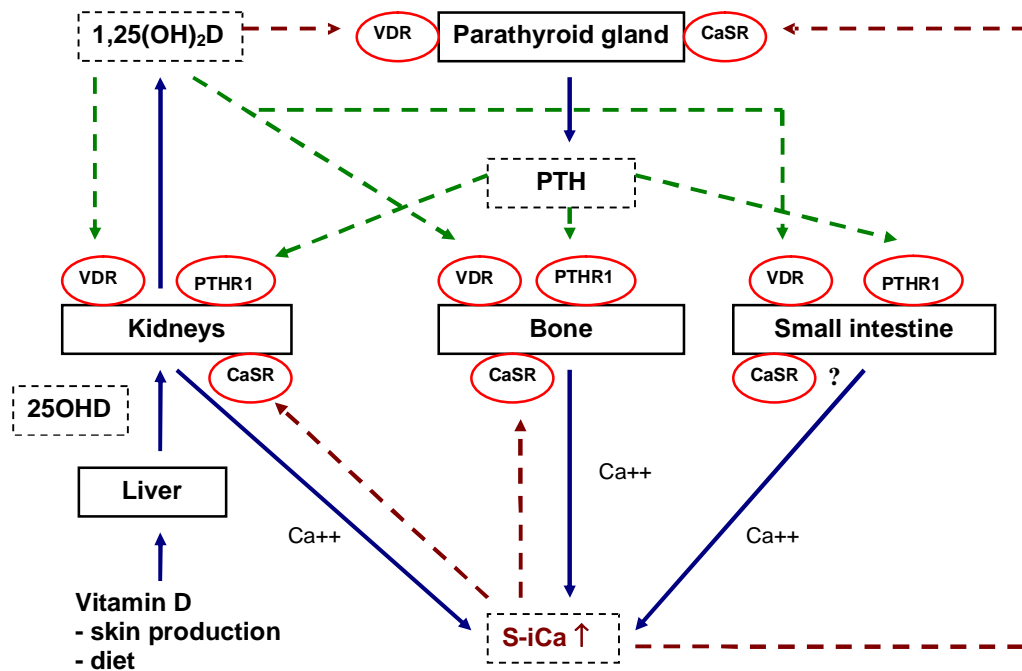


Figure 2 Calcium homeostasis is regulated by the actions of PTH and 1,25(OH)₂D in the kidneys, bone and intestines.

Abbreviations:

Ca⁺⁺ = calcium cation

S-iCa = serum ionized calcium concentration

PTH = parathyroid hormone

25OHD = 25-hydroxy-vitamin-D

1,25(OH)₂D = 1,25-dihydroxyvitamin D

CaSR = calcium sensing receptor

VDR = vitamin D receptor

PTHr1 = parathyroid hormone type I receptor

—→ = secretion, excretion

- - -→ = activation, stimulation

- - -→ = inhibition, negative feedback

The parathyroid cells sense reductions of as little as a few percent in the serum ionized calcium concentrations, which elicits relatively large increases in PTH secretion (Brown 1999). In the kidney, PTH induces the conversion of 25-hydroxy vitamin D (25OHD) into the active vitamin D metabolite 1,25(OH)₂D (Kawashima et al. 1981). PTH and 1,25(OH)₂D act synergistically to enhance mobilization of Ca²⁺ from bone into serum, and the increased 1,25(OH)₂D concentrations enhance the intestinal absorption of calcium (Weaver and Heaney 2006). In the kidney, the reabsorption of calcium in the cortical thick

ascending limbs, distal convoluted tubules and connecting tubules is stimulated by PTH and $1,25(\text{OH})_2\text{D}$ (Lajeunesse et al. 1994). In addition, PTH is suggested to have a direct action in the intestine by enhancing calcium uptake (Nemere and Larsson 2002). These actions lead to increased calcium absorption and reduced urinary calcium loss, and an increase of serum ionized calcium to the normal level. As a negative feedback mechanism, an increased $1,25(\text{OH})_2\text{D}$ concentration and a rise in the serum calcium cause a decrease in PTH secretion (Brown et al. 1995). In infants and children, calcitonin is released from the thyroid gland in response to a rise in serum calcium and signalling from gut hormones, which stops calcium release from bone as a defence mechanism against hypercalcemia (Weaver and Heaney 2006). In adults, calcitonin has little impact on calcium homeostasis because calcium absorption is lower and the extracellular fluid volume is larger.

The effects of changing extracellular ionized calcium concentrations on the parathyroid gland and the kidneys are mediated by calcium-sensing receptor (CaSR) - a G protein-coupled cell-surface glycoprotein that binds calcium. CaSR was initially cloned from the parathyroid gland (Brown et al. 1993), but was subsequently identified in tissues that participate in the regulation of calcium homeostasis, including calcitonin-secreting C cells of the thyroid gland, kidney cells (Riccardi et al. 1996), osteoblasts and osteoclasts (House et al. 1997, Kameda et al. 1998) and intestinal cells (Chattopadhyay et al. 1998); however, a role for CaSR in the intestine has not been demonstrated. Activation of CaSR by increasing extracellular ionized calcium concentrations stimulates calcium mobilization from intracellular stores and increases calcium influx through calcium channels in the cell membrane (Chen and Goodman 2004). In the parathyroid gland, an increment in intracellular calcium concentrations decreases PTH release. In kidney, the increased tubular calcium concentration activates CaSR and reduces calcium reabsorption (Bai and Friedman 2004). In bone, CaSR promotes osteoblastic differentiation and bone formation and inhibits the bone-resorbing activity of osteoclasts (see Yamaguchi 2008). The effects of $1,25(\text{OH})_2\text{D}$ are mediated through vitamin D receptor (VDR), which is present in particularly high concentrations in bone, kidney and intestine. Vitamin D receptor controls gene expression by binding the ligand, heterodimerising with retinoid X receptor, binding to vitamin D responsive elements in the promoter region of the target genes, and recruiting other transcription factors. The actions of PTH are mediated through the parathyroid hormone type I receptor (PTH1R), which is a G protein-coupled receptor. Stimulation of cells expressing PTH1R can activate two signalling systems: the adenylyl cyclase and the phospholipase C pathways (Mannstadt et al. 1999). PTH1R is expressed predominantly in osteoclasts and kidney cells, and the expression is upregulated by $1,25(\text{OH})_2\text{D}$ (Sneddon et al. 1998).

Calcium absorption

Intestinal calcium absorption is a crucial control system in the regulation of calcium homeostasis, since it provides calcium to the extracellular compartment. At lower calcium intake levels, vitamin D-dependent transcellular calcium absorption plays a major role in calcium homeostasis (Bronner et al. 1986, Pansu et al. 1993). Transcellular calcium absorption involves three steps: first, apical calcium entry through calcium channels (Hoenderop et al. 1999), secondly, cytosolic diffusion that is facilitated by vitamin D-dependent calcium binding proteins (calbindins), and finally, calcium export across the basolateral membrane by an ATP-driven mechanism. Previously, the intracellular diffusion of calcium was considered to be the critical step for vitamin D-dependent calcium absorption, yet recently it has been suggested that apical entry might also be regulated by vitamin D (Fleet et al. 2002). At higher calcium intake levels, the passive paracellular pathway is suggested to be predominant, and calcium absorption is driven by the luminal calcium concentration (Sheikh et al. 1988). Calcium absorption efficiency has been reported to depend on race (Kung et al. 1998), body weight (Wolf et al. 2000), age and estrogen status at menopause (Heaney et al. 1989), calcium intake (Heaney et al. 1989, Wolf et al. 2000), and vitamin D status (Heaney et al. 2003). Furthermore, calcium bioavailability is modulated by several life style and dietary factors, such as food source of calcium (Weaver et al. 1999), caffeine intake (Heaney 2002), lactose intake (Obermayer-Pietsch et al. 2007), protein intake (Kerstetter et al. 1998 and 2005), fat and fibre intakes, physical activity and alcohol consumption (Wolf et al. 2000, Waugh et al. 2008).

Calcium excretion

The kidneys have a limited capacity to excrete calcium, and more than 98% of calcium is reabsorbed (Lemmann 1993). Most of the filtered calcium is reabsorbed in the proximal tubules and the thick ascending limb of Henle's loop, mainly by a passive paracellular mechanism that is driven by a transepithelial electrochemical gradient generated by sodium and water reabsorption (Hoenderop et al. 2000 and 2002). About 15% of filtered calcium is reabsorbed in the distal convoluted tubules in an active transcellular process that is regulated by PTH, $1,25(\text{OH})_2\text{D}$ and calcitonin and involves apical calcium channels and intracellular calbindin proteins (see Friedman and Gesek 1995). Urinary calcium loss has been shown to increase with acidogenic diets and to decrease with alkaline diets, yet a recent review suggests that this mechanism of perturbing urinary calcium excretion does not affect net calcium retention (Bonjour 2005). Previously it was also assumed that increased protein intake has a negative impact on calcium homeostasis (Sellmeyer et al. 2001); in contrast, a more recent study reported that increased protein intake enhances calcium absorption, offsetting increased urinary losses (Kerstetter et al. 2005). Numerous experimental studies suggest that high sodium intake increases urinary calcium excretion,

which can have deleterious effects on bone remodelling. However, epidemiological studies have failed to show an association between sodium intake and BMD (Heaney 2006).

2.2.2 Recommendations and intake of calcium

Calcium functions as a threshold nutrient, since calcium retention in the body improves as calcium intake rises, but above a threshold intake further increase in intake does not alter retention (Heaney 2007). Dietary recommendations for calcium are determined by the needs of bone development and maintenance, which vary throughout the life span and with physiological status such as pregnancy and lactation. Although the data for establishing calcium requirements is not fully consistent, the current European, British and American recommendations represent a consensus that calcium intakes of 350 - 800 mg/d in childhood and 800 - 1300 mg/d in all ages after childhood are needed to ensure skeletal health (Lanou et al. 2005). The Finnish Nutritional Recommendations for calcium intake are 540 mg/d for infants, 600 mg/d for children aged 1 to 5 years, 700 mg/d for children aged 6 to 9 years, 900 mg/d for adolescents and young adults aged 10 to 20 years, and 800 mg/d for adults and the elderly (The Finnish Nutrition Recommendations 2005). In the National FINDIET 2007 Survey the average calcium intake in adult Finns is well above the recommended level (1007 mg/d in females and 1202 mg/d in males), and approximately two-thirds of dietary calcium is derived from milk and milk products (Paturi et al. 2008). In addition, several studies suggest that in the majority of Finnish children and adolescents, calcium intakes meet the recommendations (e.g. Lyytikäinen et al. 2005, Lehtimäki et al. 2006, Ruottinen et al. 2008).

2.2.3 Bone strength and remodelling

Definition and measurement of bone strength

Bone strength depends on the structural and material properties of bone, which are influenced by the rate of bone remodelling (Felsenberg and Boonen 2005). The structural properties of bone strength include bone size and geometry and the microarchitecture of bone tissue, such as trabecular architecture and cortical thickness. For example, vertebral bone size has been shown to be smaller in women with osteoporotic spinal fractures (Silva and Gibson 1997), and femoral neck strength is suggested to be a function of the hip axis length (Duan et al. 1999). With respect to geometry, for the same BMD, the further away from the centre the bone mass is distributed, the better the bone resists bending and torsion

(Bouxsein 2003). Furthermore, disconnected and thin trabeculae weaken the trabecular bone tissue (Parfitt 1992), and cortical porosity appears to increase the risk for fractures (Bell et al. 2000). The mineral and collagen composition and microdamage determine the material properties of bone. Higher bone mineral content leads to greater stiffness and compressive strength of the bone (Follet et al. 2004). The amount of collagen crosslinks is reduced in individuals with osteoporosis, leading to a reduction in material strength of the bone (Oxlund et al. 1996). The bone remodelling rate affects the ability of bone to repair microdamage, thus accelerated bone remodelling results in less mineralized bone (Ciarelli et al. 2003).

The epidemiological evidence shows that the fracture risk increases continuously with decreasing BMD without a specific threshold below which fracture risk starts to increase (Hui et al. 1988, Wasnich et al. 1989, Cummings et al. 1993, Marshall et al. 1996). Therefore, the standard tool for evaluating bone strength is BMD measurement, which is widely used in clinical assessment and in research because it is easily measured. The most commonly used techniques for BMD measurement include dual energy x-ray absorptiometry (DXA), quantitative ultrasound (QUS) and peripheral quantitative computed tomography (pQCT).

Dual energy x-ray absorptiometry

DXA is considered the gold standard for BMD measurements (Singer 2006). DXA measures the areal bone mineral density (aBMD, g/cm^2), which is then compared with the sex-matched young adult mean to yield the T-score and with age and sex-matched mean to yield a Z-score. DXA can be used to measure bone mineral content (bone mass, BMC) and BMD at any site, but the preferred sites for the diagnosis of osteoporosis are the axial sites such as the lumbar spine (posterior-anterior, from 1st to 4th vertebra) and the hip (Lewiecki et al. 2004). Peripheral DXA densitometers are used to measure BMD at the forearm or heel, and most often the measurement site is the distal radius because it contains both trabecular and cortical bone. The advantage of peripheral DXA is that the cost of the measurement is lower than for axial DXA measurement. The typical *in vivo* precision of axial DXA measurement is 1% at the spine and varies between 1.2 and 3.0% at the proximal femur (Levis and Altman 1998). The precision error for peripheral DXA is 1-3% (Cummings et al. 2002). Peripheral DXA is predictive of the overall fracture risk, yet is less predictive and accurate than BMD at the hip or spine for hip and vertebral fractures (Siris et al. 2001). A peripheral DXA measurement that indicates low BMD is not sufficient for the diagnosis of osteoporosis but can be used to identify osteoporotic patients when accompanied by a careful risk assessment (Lewiecki et al. 2004).

Quantitative ultrasound

The transmission of ultrasound through bone tissue depends on its density and structure, which can be assessed quantitatively based on the speed of sound (SOS) as the sound waves pass through bone tissue and the pattern of absorption, known as broadband ultrasound attenuation (BUA). The velocity of ultrasound waves in the bone tissue depends on the structural elasticity of the trabecular bone, and is linearly dependent on BMD (Gibson 1985). As ultrasound waves propagate through the medium, the amplitude is reduced due to absorption and scattering of the energy, and the combination of these is measured as ultrasound attenuation. The measured total ultrasound attenuation is believed to reflect some aspects of trabecular architecture and BMD, although it is difficult to distinguish which part of the variance in attenuation is attributed uniquely to BMD or to architecture (Kaczmarek et al. 2000, Nicholson et al. 2001, Cortet et al. 2004). However, ultrasound attenuation increases as porosity of bone increases, reaching a peak at porosity values of roughly 70% of the average for young adults, and then declining when porosity increases further (Njeh et al. 2001). This phenomenon is hypothesized to be caused by increased scattering of the ultrasound, and as the porosity increases, attenuation eventually reaches a peak and begins to fall. Because the association between bone architecture and QUS is complex to interpret, QUS measurements should be considered as indicators of BMD. The correlation coefficients between BUA or SOS and BMD measured with DXA range from 0.34 to 0.83 (Gregg et al. 1997, Njeh et al. 1997, Prins et al. 1998). The low correlations may result from differences in anatomic structures at the different measurement sites or in the precision of the measurement techniques (Njeh 2001). The correlation between QUS and BMD has been shown to increase when the measurement sites are closely matched (Glüer et al. 1992). The precision for QUS has been reported to range from 3% - 4% (Cummings et al. 2002). Prospective studies have shown that QUS predicts hip fracture both in elderly women (Porter et al. 1990, Hans et al. 1996, Bauer et al. 1997) and in early postmenopausal women (Huopio et al. 2004) who have a low BMD of the hip as measured by DXA. Some evidence suggests that the combination of calcaneal ultrasound attenuation and femoral neck BMD predict fracture risk better than either measurement alone (Hans et al. 1996, Bauer et al 1997).

Peripheral quantitative computed tomography

The pQCT technique is increasingly used in research since it has several advantages over DXA measurements. Firstly, pQCT measures the volumetric bone mineral density (vBMD, g/cm^3) and is therefore independent of bone size. Secondly, pQCT can separate cortical and trabecular bone tissues based on their different density values. This capacity is advantageous when investigating responses to life-style factors or therapeutic treatments that affect cortical and trabecular bone differently. Moreover, pQCT also provides

information on the geometric parameters of bone strength such as bone cross-sectional area and cortical thickness. However, pQCT is not used for diagnosis of osteoporosis due to a lack of correlation with fracture risk (Edwards et al. 2004). So far, pQCT has been almost exclusively applied at the distal radius, but the newer pQCT scanners can also measure tibia and femur, which provide a better prediction of the hip fracture risk. Compared to the DXA method, pQCT suffers from poorer precision that ranges on average from 2-4% (Cummings et al. 2002), and is higher for trabecular than for cortical bone locations (Groll et al. 1999). An additional challenge in pQCT measurements is the requirement for optimal age-specific threshold values for bone tissue compartments, which are needed for the correct assessment of bone geometry (Ward et al. 2005).

Other bone measurement techniques

Magnetic resonance imaging (MRI) of bone tissue is based on the different magnetic properties of bone and bone marrow that cause a distinct loss of signal which is proportion to the density and the spatial architecture of each tissue (Levis and Altman 1998). MRI cannot measure BMD, but it provides information on bone content and structure, such as morphological changes in the trabecular microarchitecture. Radiographic absorptiometry compares the density of the proximal phalanges to that of a wedge of aluminium that has known densities and is placed on the film alongside the hand (Yang et al. 1994). Radiographic absorptiometry has been shown to predict hip, wrist and vertebrae fracture risk in elderly women (Bouxsein et al. 2002).

Bone remodelling

Bone tissue undergoes continuous remodelling, where bone formation and bone resorption alternate and are tightly coupled to each other (Seibel 2005). The bone resorbing cells, osteoclasts, secrete lysosomal and non-lysosomal enzymes and produce a cavity on the trabecular bone surface or a cutting cone within the cortical bone from which resorption occurs. After a delay, the bone forming cells, osteoblasts, synthesize collagen and non-collagenous matrix proteins, and fill the cavity with new bone tissue that undergoes mineralization. Provided that the volumes of bone removed and replaced within the basic multicellular unit (BMU) are the same, bone remodelling is in balance and no net bone loss or structural damage occurs. During growth, bone balance in the BMU is positive, so that each remodelling event deposits bone, whereas with age bone formation decelerates and resorption accelerates, resulting in bone loss.

Bone remodelling balance is regulated by many hormones such as PTH, sex hormones, thyroid hormone, growth hormone, and by factors such as insulin-like growth factor I,

calcitonin and vitamin D, as well as local mediators in bone tissue such as cytokines and growth factors (Zaidi 2007). Trabecular bone has ten times greater surface-to-volume ratio than cortical bone, and the metabolic activity of bone tissue is accounted for predominantly by trabecular bone (Baron 1993). Cortical bone comprises 80% of the bone mass, yet it has mainly mechanical and protective functions. Strong evidence suggests that a high rate of bone remodelling is associated with bone loss, and that a long-term imbalance in bone turnover leads to bone fragility (Seibel 2006).

In recent years, several cellular and extracellular components of the skeletal matrix have been isolated and characterized, providing for the development of bone turnover markers that can be measured either from serum or urine samples (Seibel 2005). Bone turnover markers that specifically reflect either bone resorption or formation are important tools in evaluating the physiology of bone metabolism and the pathophysiology of osteoporosis, and may be used in combination with other risk factors in defining fracture risk. The markers of bone resorption are mostly products of collagen breakdown such as collagen cross-links and telopeptides, but matrix proteins such as sialoprotein and osteoclast-related enzymes such as tartrate-resistant acid phosphatase also reflect the rate of bone resorption. Markers of bone formation are either precursor molecules of procollagen (propeptides of type I collagen), proteins that have a role in osteoblast function (osteocalcin) or proteins that play an important role in osteoid formation and mineralization (alkaline phosphatase). Serum total calcium concentration reflects changes in the function of the parathyroid gland. For instance, hypercalcemia can be a sign of hyperparathyroidism, which causes increased bone resorption and bone loss (see Åkerström et al. 2005).

Most bone remodelling markers exhibit significant variability due to subject-related biological or sample handling-related technical factors (Seibel 2005). Subject-related variables include age, gender, ethnicity, recent fractures, pregnancy or lactation, medications (e.g. oral contraceptives, glucocorticosteroids), diseases (e.g. renal impairment, liver disease), immobility, diet, exercise, and diurnal, menstrual and seasonal variation. The technical sources of variability include the choice of sample (urine vs. serum), mode of sample collection (24-hour collection vs. morning void), preparation of the subject (e.g. fasting, exercise), and the correct handling, processing and storage of the sample.

2.2.4 Skeletal life span

Childhood and adolescence

Skeletal growth in the fetus and infant is rapid, and in early childhood bone mass attenuation is slow until it accelerates in puberty (Heaney et al. 2000). Incorporation of bone mineral increases five-fold during pubertal maturation, which on average occurs in girls between ages 11 and 14 and in boys between ages 13 and 17 (Theinz et al. 1992). About 37% of total bone mass can be attained in puberty between Tanner stages 2 and 4, and approximately 85-90% of final adult bone mass is acquired by the age of 18 years in girls and 20 in boys (Bonjour et al. 1991, Matkovic et al. 1994). The age at which PBM is attained is debated, and estimates range from late adolescence (Bonjour et al. 1991, Theintz et al. 1992, Lu et al. 1994, Bonjour et al. 2001) to the third decade (Recker et al. 1992, Teegarden et al. 1995) or up to the fourth decade (Krolner and Pors Nielsen 1982, Rodin et al. 1990, Arlot et al. 1997). During growth in childhood and adolescence, bone mass increase results from growth in both bone length and diameter. In the prepubertal years, appendicular growth velocity is more rapid than axial growth velocity, until at puberty when appendicular growth slows and axial growth accelerates (Tupman 1962). Several studies indicate an association between chronological age and BMC at the lumbar spine, femoral neck and the entire skeleton (see e.g. Gilsanz 1998). At puberty, the increase in bone size occurs before the increase in BMC (Mølgaard et al. 1999), however, DXA values at the radius have not been found to be affected by puberty (Zanchetta et al. 1995). Thus, in adolescence, BMC is a more reliable measurement of bone strength than BMD.

Sex differences in bone diameter are established during peripubertal growth (Seeman 2001). In boys, androgens, growth hormone, and insulin-like growth factor I stimulate periosteal bone apposition that is accompanied by less endosteal apposition, which results in enlargement of total and medullary diameters of the bone along with cortical thickening. In girls, estrogen inhibits periosteal apposition and stimulates endosteal apposition, which leads to greater cortical thickness and a narrower medullary cavity. Thus, boys build a longer and wider long bone than girls, yet the cortex is only slightly thicker in boys and the volumetric BMD does not differ between the sexes (Zamberlan et al. 1996). However, long bones in boys have greater strength in bending because the periosteal apposition places the bone mass further from the neutral axis (Turner and Burr 1993). At puberty, trabecular BMD increases due to thickening of the trabeculae but the trabecular numbers do not increase since they are determined at the growth plate (Parfitt et al. 2000). Trabecular thickening is comparable between sexes but is greater in blacks than in whites (Gilsanz et al. 1991).

Adulthood and aging

As longitudinal growth comes to completion, bone mass is commonly believed to remain relatively stable, and if there is loss of bone, the loss is slow because of the slow bone remodelling rate (Seeman 2004). Many cross-sectional studies have shown that bone mass is lost in women during the premenopausal years (Buchanan et al. 1988, Rodin et al. 1990, Sowers et al. 1991, Ravn et al. 1994, Arlot et al. 1997, Riggs et al. 2004). Results from several prospective studies suggest that trabecular bone is most susceptible to premenopausal bone loss (Krolner and Pors Nielsen 1982, Riggs et al. 1986, Citron et al. 1995, Prior et al. 1996, Uusi-Rasi et al. 2007, Riggs et al. 2008). However, a few studies have produced contradictory results, and longitudinal assessments suggest relatively stable bone mass levels (Mazess and Barden 1991, Recker et al. 1992, Sowers et al. 1998, Warming et al. 2002). Bone loss in young men has not been studied as comprehensively as in women but a few studies suggest bone loss also occurs for men in young adulthood (Aaron et al. 1987, Kalender et al. 1989, Riggs et al. 2004 and 2008). In perimenopausal women, unbalanced bone remodelling starts with a decrease in bone formation whereas after menopause the accelerated bone loss is mainly due to an increase in bone resorption (Seifert-Klauss et al. 2002). In aging men, osteoclast function remains largely constant, and decreased bone formation seems the principal factor in bone loss (Seeman 2004).

It is noteworthy that most studies of bone loss are carried out using bone densitometry that offers information on the mineral content of bone tissue but ignores the structural basis of bone loss. Nevertheless, after attainment of PBM, structural properties of bone continue to change (Duan et al. 2001, Duan et al. 2003). Aging is associated with enlargement of bones because bone is resorbed on the endocortical and trabecular surfaces but is simultaneously formed on the periosteal surface, resulting in thinning of the cortex and widening of the bones. Periosteal bone formation is greater in aging males than in females, which accounts for the wider bones in males. Women and men lose the same amount of trabecular bone but the patterns of trabecular bone loss differ between the sexes (Aaron et al. 1987, Kalender et al. 1989). Trabecular bone loss in women is explained by an increased resorption, with losses of trabecular numbers and connectivity, whereas in men, reduced bone formation causes trabecular thinning. Later in life, cortical bone loss accelerates in both sexes (Seeman 2004). Women have greater cortical bone loss and increased cortical porosity, whereas men lose less cortical bone due to greater periosteal apposition.

2.2.5 Osteoporosis and its risk factors

Definition and prevalence of osteoporosis

The concept of osteoporosis as a clinical problem was first recognized over 150 years ago by an English surgeon, Sir Astley Cooper, who noted that hip fractures might result from age-related reduction in bone mass or quality (Cooper 1999). In 1940 an American physician and endocrinologist Fuller Albright described postmenopausal osteoporosis as a consequence of impaired bone formation due to estrogen deficiency (see Raisz 2005). The current concept defines osteoporosis as a bone disease characterized by low bone mass and the microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fractures (Anon. 1993). The World Health Organization defines osteoporosis as a BMD value of -2.5 standard deviation (SD) or lower in comparison to young adults (WHO 1994). Fracture risk approximately doubles with each SD reduction in the T-score of BMD, irrespective of fracture type and site of BMD measurement (Marshall et al. 1996). Osteoporosis is classified into primary and secondary osteoporosis (Riggs et al. 1982). The first type of primary osteoporosis (postmenopausal osteoporosis) is associated with reduced estrogen levels at menopause and affects mainly trabecular bone tissue. The second type (senile osteoporosis) is prevalent at an older age in both sexes, and is characterized by age-related deterioration of both trabecular and cortical bone structures. Secondary osteoporosis is caused by other diseases or medications such as anorexia nervosa, malabsorption syndrome, primary hyperparathyroidism, transplantation surgery, chronic renal failure, hyperthyroidism, Cushing's syndrome or glucocorticoid therapy.

According to the International Osteoporosis Foundation, osteoporosis affects about 75 million people in Europe, the USA and Japan (EFFO and NOF 1997). It has been estimated that one in three women over 50 years of age will experience osteoporotic fractures, as will one in five men (Melton et al. 1992, Melton et al. 1998, Kanis et al. 2000). In Finland, the age-adjusted hip-fracture incidence showed a steady increase between 1970 and 1997, but the recent epidemiologic data indicate that the hip-fracture rate has declined by 17% in women and by 6% in men between 1997 and 2004 (Kannus et al. 2006). The authors suggested that this may be due to a cohort effect toward a healthier aging population and improved functional capability among older people, higher average body weight, or may result from actions to prevent osteoporosis and fractures.

Risk factors for osteoporosis and fractures

Increased susceptibility to osteoporosis results from a variety of risk factors that cause a failure in attainment of optimal PBM during growth or unbalanced bone remodelling later in life. Some risk factors of osteoporosis contribute to fracture risk independently of BMD (Kanis 2002), therefore, clinical risk factors may be used to identify individuals at a high risk for fractures (Table 2).

Table 2. Risk factors for osteoporotic fractures (modified from Kanis 2002).

Risk factors that predict low BMD	Risk factors that contribute to fracture risk independently of BMD
Female sex	Age
Asian or white ethnic origin	Previous fragility fracture
Premature menopause	Family history of hip fracture
Primary and secondary hypogonadism	Glucocorticoid therapy
Disease or medication associated with secondary osteoporosis	High bone remodelling rate
Inadequate calcium intake	Neuromuscular disorders
Vitamin D deficiency	Poor visual acuity
Low level of physical activity and long-term immobilization	Low bodyweight
Excessive alcohol consumption	Cigarette smoking

In both women and men, increasing age and low BMD are the most important risk factors for osteoporotic fractures (van der Klift et al. 2005), yet there is more evidence connecting low BMD with increased fracture risk in women than in men. The incidence of osteoporosis is lower in men than in women due to greater bone mass, bone size, shorter life span, and the absence of equivalent male menopause (Scane et al. 1993). Although the lifetime risk of hip fracture in men is lower than in women, in men the mortality rate from hip fracture is almost twice that of women (Campion and Maricic 2003). Bone mass is known to differ between ethnic groups (Pollitzer and Anderson 1989). Blacks have higher bone mass and lower incidence of fractures in comparison to whites. The Chinese and Japanese have significantly less cortical bone than whites, which imposes on them a higher risk of fractures.

The risk for fragility fractures increases when there is a prior fracture (Kanis et al. 2004a), and a parental risk of hip fracture confers on offspring an increased risk of fracture independent of BMD (Kanis et al. 2004b). In postmenopausal women, low serum estradiol levels are associated with low BMD but also with increased risk of vertebral fractures

independent of BMD (Goderie-Plomp et al. 2004). Interestingly, in elderly men, a decline in estradiol level is more strongly correlated with low BMD than a decline in testosterone level (Goderie-Plomp et al. 2004). Long-term glucocorticoid therapy increases the risk for osteoporosis and fractures, and bone loss is most rapid in the first few months of treatment (Kanis et al. 2004c). Results from case-control studies have shown an increased risk of fractures with several disorders or medications that cause secondary osteoporosis, a high bone remodelling rate and susceptibility to falling (Kanis 2002).

The current consensus is that adequate calcium intake is an important determinant of PBM attainment (see Flynn 2003), and calcium supplementation enhances bone accretion during growth (Matkovic et al. 2005); however, the optimum dietary intake of calcium is not well defined. Interestingly, a recent re-evaluation of the epidemiological and prospective findings suggested that the existing available data do not provide evidence of a beneficial effect of increasing the consumption of milk and other dairy products on bone health in children and young adults (Lanou et al. 2005). Nonetheless, latest meta-analysis that combined data from randomized controlled trials and observational studies showed that increased dietary calcium or dairy products significantly increases total body and lumbar spine BMC and BMD in children with low base-line intakes (Huncharek et al. 2008). Sufficient calcium intake is crucial for maintaining bone mass in women before menopause (Welten et al. 1995) and for attenuating bone loss during menopause and later in life (Shea et al. 2004). Several randomized controlled trials have shown beneficial effects of calcium supplementation on BMD in the decades following menopause (see Flynn 2003, Shea et al. 2004).

The findings from the observational epidemiological studies indicate that the association between low dietary calcium intake and fractures is inconsistent (see e.g. Cummings et al. 1997). A meta-analysis of almost 40 000 people from six prospectively-studied cohorts suggested that a self-reported low intake of milk is not associated with any marked increase in fracture risk (Kanis et al. 2005a). Another recent meta-analysis of prospective cohort studies and randomized controlled trials concluded that calcium intake is not significantly associated with fractures in either sex and that calcium supplementation is not beneficial in preventing hip fractures (Bischoff-Ferrari et al. 2007). In any case, the evidence regarding the effects of calcium intake on bone health is not comprehensive since research to determine levels of calcium that are necessary for adequate nutrition is lacking and the effects of calcium supplementation may not be long-term. Moreover, the number of studies in men is inadequate.

Vitamin D deficiency, as determined by low serum 25OHD concentrations, decreases PBM accrual in adolescents (Outila et al. 2001, Lehtonen-Veromaa et al. 2002, Välimäki et al. 2004, Viljakainen et al. 2006) and increases the risk of fractures in the elderly

(Dawson-Hughes et al. 2005). A recent meta-analysis indicated that calcium supplementation alone or in combination with vitamin D is effective in the preventive treatment of osteoporotic fracture in people aged 50 years and older (Tang et al. 2007). Furthermore, vitamin D supplementation is thought to decrease the risk of falls and fractures in the elderly people (Bischoff-Ferrari et al. 2005 and 2006). Consistent evidence suggest that vitamin D plays a significant role in regulating muscle strength by promoting protein synthesis and growth of muscle fibers via highly specific vitamin D receptors in the skeletal muscle, thus decreasing the risk of falls and fractures (see Boonen et al. 2006).

There is strong evidence that physical activity during growth enhances bone accrual (MacKelvie et al. 2002, 2003 and 2004), and the effects of exercise on bone are most pronounced during the most rapid growth peak (Kannus et al. 1995, Lehtonen-Veromaa et al. 2000). The significance of physical activity during adulthood is mainly to maintain bone mass and prevent bone losses; however, some evidence suggest that high-impact exercise may improve BMD in adults (Wolff et al. 1999). Among the elderly, the level of physical activity has an inverse and dose-dependent relationship with the incidence of fractures (Joakimsen et al. 1997). The immobilization reduces bone mass (Järvinen and Kannus et al. 1997) because it leads to a negative calcium balance due to decreased bone formation and intestinal calcium absorption, and increased bone resorption and urinary calcium excretion (Le Blanc et al. 1995, Smith et al. 1999, Zittermann et al. 2000).

A low body mass index ($<20 \text{ kg/m}^2$) and low body weight are associated with low BMD and increased fracture risk (van der Klift 2005). In men, lean body mass contributes significantly to BMD, whereas both lean body mass and fat mass contribute equally to BMD in women (Lim et al. 2004). Three meta-analyses have shown that smoking increases fracture risk, especially at the hip, in both women and men, and that BMD appeared to have only a small effect on smoking-associated fracture risk (Law et al. 1997, Ward and Klesges 2001, Kanis et al. 2005b). A study combining data from three large prospective cohorts showed that alcohol intake of more than 2 portions per day was associated with a higher risk for fractures (Kanis et al. 2005c).

2.3 Skeletal genetics

2.3.1 Inheritance of calcium homeostasis, bone strength and fractures

Calcium homeostasis and bone strength are complex traits that do not exhibit the classic Mendelian recessive or dominant inheritance patterns attributable to a single gene locus (Lander et al. 1994). Thus, they are polygenetically regulated and each gene contributes only to a small effect that is difficult to detect even in studies with large numbers of individuals (Gueguen et al. 1995). Hunter et al. (2001) has shown that the genetic contribution to bone remodelling, calcium excretion, and hormonal regulation of bone and calcium metabolism is substantial. This classical twin study showed that heritabilities were 54-65% for S-PTH, 28-57% for S-25OHD, 53-74% for S-1,25(OH)₂D, 56-66% for serum concentration vitamin D binding protein, 14-80% for markers of bone formation and resorption, e.g. 67-80% for bone-specific alkaline phosphatase (BSAP), 14-44% for osteocalcin, 52-64% for deoxypyridinoline (DPD), and 41-61% for calcium excretion. Numerous twin and family studies suggest that genetic factors may account for 50% to 85% of the inter-individual variance in PBM, depending on the bone site, the sex and the age of subjects studied (Smith et al. 1973, Pocock et al. 1987, Krall and Dawson-Hughes 1993, Cummings et al. 1995). However, most twin studies on genetic variation of BMD have been small in size and mostly involved women. A recent twin study on 300 hundred Finnish men showed that heritability of BMD was 75% at the femoral neck and 83% at the lumbar spine (Videman et al. 2007). Moreover, evidence suggests high heritability estimates for bone geometry and structure phenotypes. For example, the genetic component of hip axis length and broadband ultrasound attenuation ranges from 53% to 62% (Arden et al. 1996, Slemenda et al. 1996).

The role of genetic factors in the pathogenesis of bone loss is less clear, and the genes that regulate bone loss might differ from those that regulate PBM (Harris et al. 1998). Twin studies present evidence both for (Kelly et al. 1993) and against (Christian et al. 1989) heritability of age-related bone loss. The age at menopause is reported to be genetically determined (Snieder et al. 1998). Furthermore, studies on genetic susceptibility to osteoporotic fractures have yielded conflicting results. A family history of fracture predicts fractures independently of BMD (Cummings et al. 1995, Torgerson et al. 1996), and the estimates of wrist fracture heritability vary from 25% (Deng et al. 2000) up to 54% (Andrew et al. 2005). In contrast, a Finnish twin study did not find evidence for heritability of fracture risk (Kannus et al. 1999). A large Swedish study showed that the

heritability of hip fracture dropped off with age (Michaëlsson et al. 2005), suggesting that the genetic contribution to fractures decreases with age as the environmental determinants of fracture risk become more important. Moreover, heredity may also contribute significantly to other determinants of osteoporotic fracture risk such as muscle strength (Arden and Spector 1997) and consumption of milk products (Obermayer-Pietch et al. 2004).

2.3.2 Genome-wide studies on skeletal genetics

Classic linkage studies on skeletal genetics have examined the relationship between a series of polymorphic microsatellite markers and the inheritance of bone mass within family members (Ralston and de Crombrughe 2006). Despite the high heritability of BMD, linkage studies have so far only identified a few loci that meet the criteria for evidence of linkage – that is, a LOD-score over 3.6 (LOD= linkage of disease)(Huang and Kung 2006). Moreover, the evidence from different linkage studies is inconsistent, which may be due to differences in enrolment criteria, study designs and genetic heterogeneity between study populations.

Recently, genetic dissection of osteoporosis has made a tremendous progress as a result of large population-based genome-wide association studies. A large genome-wide association study in a population-based sample, the Framingham Heart Study, revealed 40 different SNPs that were associated with several bone phenotypes including BMD at the femoral neck, trochanter, and lumbar spine (Kiel et al. 2007). Interestingly, 50-75% of the SNPs that were associated with bone phenotypes were located in or near genes that had not previously been studied in relation to osteoporosis. This study showed LOD-scores ≥ 3 on chromosomes 15 and 22 for femoral bone geometry phenotypes. Ioannidis and co-workers (2007) have performed a meta-analysis of genome-wide association studies in which spine and hip BMDs were studied. Loci in chromosomes 1p, 1q, 3p, 11p, 11q, 12q, 18p and 18pter showed a significant linkage for the lumbar spine BMD, and the most significant linkage was found for the genetic location 1p13.3-q23.3 in women but not in men. The potential candidate genes for osteoporosis that have been identified at these locations include, for example, the genes for tissue specific alkaline phosphatase, cathepsin K, methylenetetrahydrofolate reductase (MTHFR), tumour necrosis factor receptor 2 (TNFR2), and leptin receptor (Ferrari 2006, Huang and Kung 2006). Seven loci in chromosomes 5q, 9p, 9q, 14q, 17p and 17q yielded significant linkage with the hip BMD. The locus 17p-q contain potential osteoporosis candidate genes such as collagen type 1 (COL1A1), sclerostin (SOST), interleukin 10 (IL-10), and growth hormone 1 (GH1). The results from a recent genome-wide association study in Icelandic subjects revealed an association between three genomic regions that are close to or within genes

previously shown to be important for bone strength (Styrkarsdottir et al. 2008). The regions that were associated with BMD of the hip and lumbar spine were 6q25, containing the estrogen receptor 1 gene (ESR1), 8q24, containing the osteoprotegerin gene (OPG), and 13q14, containing the receptor activator of the nuclear factor- κ B ligand gene (RANKL). In addition, the Icelandic study showed a novel association between BMD and the genomic regions which are close to the zinc finger and BTB domain containing 40 gene (ZBTB40)(1p36), and the major histocompatibility complex region (6p21). These associations were confirmed in the replication sets of Icelandic, Danish and Australian subjects. Another recent genome-wide association study with participants of white European ancestry suggested that two SNPs near the osteoprotegerin gene and in the lipoprotein-receptor-related protein gene (LRP5) are strongly associated with risk of osteoporosis and osteoporotic fractures, and the combined effects of these risk alleles is similar to that of most well-replicated environmental risk factors (Richards et al. 2008).

2.3.3 The candidate gene approach in skeletal genetics

The candidate gene approach in osteoporosis research has focused on genes that encode calciotropic hormones and their receptors, bone matrix proteins, local regulators of calcium and bone metabolism and other genes that have been found to be associated with BMD or osteoporotic fractures (Table 3). Genes coding for the lactase enzyme, VDR, CaSR and PTH are examples of candidate genes for osteoporosis that are involved in the regulation of calcium homeostasis and may thus contribute to bone strength. Evidence for associations between these genetic variants and indicators of calcium homeostasis and bone strength is introduced in detail in this thesis.

Table 3. Examples of candidate genes shown to be associated with calcium homeostasis, bone mineral density and bone remodelling or osteoporosis and fractures in at least four independent association studies (Zajickova and Zofkova 2003, Huang and Kung 2006, Lei et al. 2007).

Candidate genes	Locus	SNPs	References
A. Calcium homeostasis			
Vitamin D receptor (VDR)	12q12-14	<i>BsmI</i> <i>ApaI</i> <i>TaqI</i>	Gong et al. 1999a*, Thakkinstian et al. 2004a*, Uitterlinden et al. 2006
Parathyroid hormone (PTH)	11p15	<i>BstBI</i>	Gong et al. 1999b, Hosoi et al. 1999, Kanzawa et al. 1999, Katsumata et al. 2002
Calcium sensing receptor (CaSR)	3q13-21	A986S R990G	Cole et al. 1999, Lorentzon et al. 2001, Eckstein et al. 2002 Corbetta et al. 2006, Scillitani et al. 2007, Vezzoli et al. 2007
Lactase-phlorizin hydrolase (LCT)	2q21-22	C/T ₋₁₃₉₁₀	Obermayer-Pietsch et al. 2004 Rasinperä et al. 2004 Gugatschka et al. 2005 Lember et al. 2006
B. Bone matrix proteins			
Collagen type 1 α (COL1A1)	17q21-22	Sp1 (intron)	Mann and Ralston 2003*
Osteocalcin (BGP)	1q25-31	<i>HindIII</i> C/T (promotor)	Chen et al. 2001 Willing et al. 2003, Kim et al. 2006 Yamada et al. 2003

Alpha2-HS-glycoprotein (AHSG)	3q27	AHSG1/AHSG2	Zmuda et al. 1998, Deng et al. 2005, Lei et al. 2005, Yang et al. 2007
<i>C. Local regulators</i>			
Transforming growth factor β 1 (TGF- β 1)	19q13	713-delC (intron 4) T/C -pro/Leu (exon1)	Langdahl et al. 1997 Yamada et al. 1999, Yamada et al. 2001, Dick et al. 2003
Insulin-like growth factor 1 (IGF-1)	12q22-24	CA repeat (promotor)	Niu and Rosen 2005*
Interleukin 6 (IL-6)	7q21	3'AT repeat G/C (promoter) CA repeat (13-18)	Murray et al. 1997 Ferrari et al. 2001, Nordström et al. 2004 Tsukamoto et al. 1999, Wang et al. 2006
<i>D. Others</i>			
Apolipoprotein E (ApoE)	19q13	E2, E3, E4	Shiraki et al. 1997, Cauley et al. 1999, Salamone et al. 2000, Dick et al. 2002
Methylenetetrahydrofolate reductase (MTHFR)	1p36	C677T A/V	Miayo et al. 2000, Jorgensen et al. 2002, Abrahamsen et al. 2003, Villadsen et al. 2005
Low density lipoprotein receptor-related protein 5 (LRP5)	11q13.4	Val667Met, Ala1330Val	Ferrari et al. 2005, Bollerslev et al. 2005, Saarinen et al. 2007, van Meurs et al. 2008

* Meta-analysis

Lactase gene polymorphisms

Genetic lactase non-persistence (adult-type hypolactasia) is a recessively inherited condition that is characterized by a developmental decline in the activity of lactase-phlorizin hydrolase in the brush border of small intestinal epithelial cells (Sahi et al. 1973, Flatz and Rotthauwe 1977). Enattah and the colleagues (2002) were the first to identify the genetic variant associated with adult-type hypolactasia by studying Finnish families. They found that lactase persistence correlates strongly with the C/T₋₁₃₉₁₀ polymorphism located 13.9 kb upstream from the lactase gene at the chromosomal site 2q21-22. *In vitro* studies suggest that the C/T₋₁₃₉₁₀ variant region is an enhancer of lactase gene expression (Olds and Sibley 2003, Troelsen et al. 2003) regulating the binding of transcription factors such as Oct-1 (Lewinsky et al. 2005). Intestinal biopsies have verified the association of the C/T₋₁₃₉₁₀ polymorphism with lactase enzyme activity (Kuokkanen et al. 2003). Expression of the lactase gene in the intestinal mucosa is several times higher for the T₋₁₃₉₁₀ allele than for the C₋₁₃₉₁₀ allele. Therefore, both the T/T₋₁₃₉₁₀ and C/T₋₁₃₉₁₀ genotypes possess sufficient enzyme activity to be classified as lactase persistent, and the C/C₋₁₃₉₁₀ genotype is classified as lactase non-persistent.

Subsequent study has revealed that the frequency of the T₋₁₃₉₁₀ variant is systematically correlated with the reported prevalence of lactase persistent among 37 studied populations (Enattah et al. 2007). However, some evidence suggests that the C/T₋₁₃₉₁₀ polymorphism on its own does not explain all the variation in lactase gene expression in Europeans (Poulter et al. 2003). Furthermore, other genetic variants of lactase persistence have been identified in close proximity to the C/T₋₁₃₉₁₀ polymorphism, for example in African populations (Mulcare et al. 2004, Ingram et al. 2007). Nevertheless, the presence of the same genetic variants of lactase persistence in distantly related populations suggests that the persistence variant arose long before differentiation of the populations (Enattah et al. 2002). Recent evidence indicates that the T₋₁₃₉₁₀ allele is of Caucasian origin and has most probably arisen independently more than once in human history, and that the convergent evolution of lactase persistence alleles is still an ongoing process (Enattah et al. 2007).

Studies in Austrian, Finnish, Swedish and Estonian populations have shown that the C/C₋₁₃₉₁₀ genotype is associated with lower consumption of milk and milk products, lower calcium intake, and impaired calcium absorption (Obermayer-Pietsch et al. 2004, Gugatschka et al. 2005, Lember et al. 2006, Anthoni et al. 2007, Obermayer-Pietsch et al. 2007, Torniainen et al. 2007). Furthermore, some Finnish studies indicate a relationship between genetic lactase non-persistence and increased prevalence of low-lactose diets in children and adolescents (Rasinperä et al. 2004, Rasinperä et al. 2006). In contrast, Finnish

studies in young men (Enattah et al. 2004), postmenopausal women (Enattah et al. 2005a), and elderly people (Enattah et al. 2005b) have not observed any differences in the consumption of milk products or calcium intake between the lactase genotypes. Obermayer-Pietsch and the colleagues (2004) were the first to report the harmful effects of genetic lactase non-persistence on BMD and fracture incidence in the Austrian population. Thus far Finnish studies have revealed inconsistent evidence for an association between genetic lactase non-persistence and bone health. The bone remodelling rate and acquisition of peak bone mass did not differ between the lactase genotypes in young men (Enattah et al. 2004), and genetic lactase non-persistence was not a risk factor for osteoporosis in postmenopausal women (Enattah et al. 2005a). However, in Finns aged 85 years and older, genetic lactase non-persistence was associated with fractures (Enattah et al. 2005b).

Vitamin D receptor gene polymorphisms

The chromosomal location of the VDR gene is 12q12-14, and approximately 62 polymorphisms have been identified across the relevant areas of the gene (Fang et al. 2005). The most extensively studied SNPs include the *BsmI*, *ApaI* and *TaqI* polymorphisms located in the 3' untranslated region (UTR), and the translation initiation start codon polymorphism *FokI* (Figure 3). The 3' polymorphisms *BsmI*, *ApaI* and *TaqI* are in strong linkage disequilibrium with each other (Morrison et al. 1994), which means that they travel as blocks, generating various haplotypes, from generation to generation. A more novel polymorphism at the Cdx-2 (an intestine-specific homeodomain-containing transcription factor) binding site in the promotor region of VDR has been identified as functionally active (Arai et al. 2001, Fang et al. 2005).

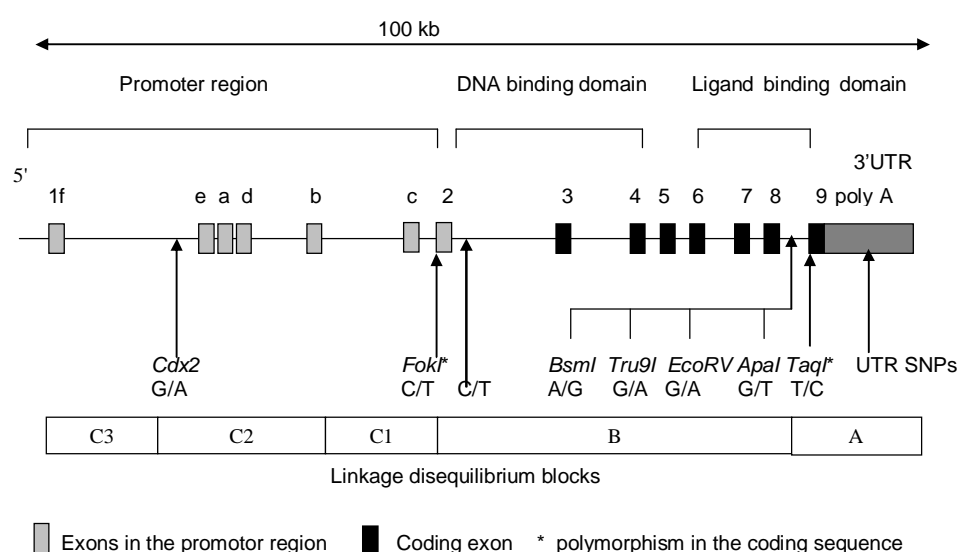


Figure 3 Positions of polymorphisms in the vitamin D receptor gene (modified from Uitterlinden et al. 2002 and Rukin and Strange 2007).

During the last decade, VDR polymorphisms have received the greatest attention among the candidate SNPs for osteoporosis, and by the end of 2006, more than 150 association studies had reported VDR gene polymorphisms and bone-related phenotypes (Liu et al. 2006). In two landmark studies Morrison and colleagues (1992 and 1994) reported a significant association between the polymorphic sites at the 3' end of the VDR gene and BMD in Caucasian twins. They concluded that up to 75% of the genetic variation in BMD could be explained by VDR gene polymorphisms; however, the same group subsequently reported problems with their original genotyping so that the heritability component of the VDR gene was lower than first reported (Morrison et al. 1997). Thereafter, evidence for the contribution of VDR gene polymorphisms to bone health has become controversial. In 1996, Cooper and Umbach published the first meta-analysis with sixteen studies on the associations between VDR polymorphisms and BMD and concluded that the *BsmI* genotypes contributed to BMD, yet the effect was weaker than reported by Morrison et al. (1994). The second meta-analysis comprised 75 studies and confirmed a strong association between the *BsmI* polymorphism and bone mass (Gong et al. 1999a). A subsequent meta-analysis found an association between the *BsmI* polymorphism and the spinal BMD in postmenopausal but not in premenopausal women (Thakkestian et al. 2004a). A large population-based study in British women examined whether *Cdx-2*, *FokI*, *BsmI*, *Apal* and *TaqI* polymorphisms influence bone health, and concluded that VDR does not seem to contribute to BMD or bone remodelling (Macdonald et al. 2006). The allelic variants of the VDR gene have not been consistently associated with rates of bone loss among postmenopausal women; however, in most studies the number of subjects was small and the follow-up time short (Zmuda et al. 2000). A meta-analysis of thirteen studies failed to show a relationship between the *BsmI* and *TaqI* polymorphisms and fracture risk (Fang et al. 2006).

Thakkestian and colleagues (2004b) were the first to apply meta-analysis to VDR haplotypes, and showed that rather than the individual genotypes, the haplotypes were significantly associated with osteoporosis. The GENOMOS project, which involves 9 European research teams, evaluated the relationship between VDR polymorphisms, BMD and fractures (Uitterlinden et al. 2006). This participant level meta-analysis concluded that the *FokI* polymorphism and *BsmI*, *Apal* and *TaqI* haplotypes were not associated with fractures, but the *Cdx2* polymorphism may be associated with vertebral fractures. Moreover, the results from two recent large prospective studies indicate that the VDR haplotypes in the 3' UTR and common C to T polymorphism in exon 2 of the VDR gene contribute to increased fracture risk in elderly women and men (Grundberg et al. 2007, Moffett et al. 2007).

The VDR polymorphisms were among the first SNPs used to test diet-gene interactions in bone health when response to treatment with calcium or 1,25(OH)₂D was studied in association with BMD (Uitterlinden et al. 2004). The *BsmI* and *FokI* polymorphisms were shown to influence calcium absorption efficiency (Wishart et al. 1997, Zmuda et al. 1997, Ames et al. 1999), and the relationship may be modified by calcium intake (Dawson-Hughes et al. 1995, Gennari et al. 1997). In contrast, some studies have failed to find any associations between the VDR genotypes and calcium absorption (Francis et al. 1997, Kinyamu et al. 1997). Furthermore, the *FokI* polymorphism was found to modulate the response of bone tissue to a calciuric diet with high sodium and protein intakes (Harrington et al. 2004). A limited number of studies have examined whether dietary calcium intake modulates the associations between the VDR genotypes and bone phenotypes, and the results have been inconsistent. A few studies suggest that the relationship between the *BsmI* polymorphism and BMD is dependent on calcium intake (Salamone et al. 1996, Kiel et al. 1997); however, contradictory results have also been published (Garnero et al. 1996). Furthermore, Ferrari and colleagues (1998) reported that the association between the *FokI* polymorphism and BMD was most evident at high calcium intakes. Follow-up studies have indicated that BMD phenotypes for the various VDR genotypes could respond differently to calcium and vitamin D supplementation (Ferrari et al. 1995, Krall et al. 1995, Matsuyama et al. 1995, Graafmans et al. 1997), yet these studies had contradictory results concerning the question of which VDR genotype is beneficial. Interestingly, caffeine intake had an interaction with the VDR genotype that was found to determine bone loss in postmenopausal women (Rapuri et al. 2001).

Calcium sensing receptor gene polymorphisms

The CaSR gene is located at chromosomal position 3q13.3-21 (Janicis et al. 1995) and spans 45 kb of genomic DNA with the coding region between exons 2 to 7 (Garret et al. 1995). Three SNPs, A986S, R990G and Q1011E are located in exon 7 of the CaSR gene, which codes for the intracellular carboxyterminal tail of the CaSR protein (Heath et al. 1996). These polymorphisms have been most studied in association with bone health, in addition to the dinucleotide CA₁₂₋₂₀ repeat polymorphism in the flanking region of the CaSR gene (Tsukamoto et al. 1998). It is evident that linkage disequilibrium exists between the A986S, R990G and Q1011E SNPs (Hendy et al. 2000). The A986S polymorphism causes replacement of the non-polar hydrophobic alanine by the polar but uncharged serine; the R990G polymorphism codes for substitution of a positively charged arginine with the polar but uncharged glycine; and at codon 1011 the polar but uncharged glutamine is replaced by the negatively charged glutamic acid (Harding et al. 2006).

Most research has focused on the A986S polymorphism and a few studies suggest that the A986S polymorphism is associated with serum total calcium concentration (Cole et al. 1999, Cole et al. 2001, Lorenzon et al. 2001, Eckstein et al. 2002). However, some results are contradictory (Young et al. 2003) and many others show no correlation (Miedlich et al. 2001, Cetani et al. 2002, Bollerslev et al. 2004, Perez-Castrillón et al. 2006). Scillitani and colleagues (2004) suggested that the haplotypes of the A986S and Q1011E loci contribute to serum ionized calcium concentration. Furthermore, the R990G polymorphism is associated with calcium excretion in patients with increased risk of kidney stones and patients with primary hyperparathyroidism (Vezzoli et al. 2002, Corbetta et al. 2006, Scillitani et al. 2007, Vezzoli et al. 2007). Although two studies suggest a relationship between the A986S polymorphism and BMD (Lorenzon et al. 2001, Eckstein et al. 2002), subsequent studies have failed to confirm these preliminary findings (Takacs et al. 2003, Cetani et al. 2003, Bollerslev et al. 2004, Perez-Castrillón et al. 2006). Studies in Japanese and Chinese populations suggest that the CA₁₂₋₂₀ repeat polymorphism is associated with BMD in postmenopausal women (Tsukamoto et al. 2000, Lau et al. 2005). Haplotype analysis of the three CaSR polymorphisms revealed no associations between haplotypes and BMD or fractures (Scillitani et al. 2007).

Parathyroid hormone gene polymorphisms

The gene for the human PTH polypeptide is located at chromosomal position 11p15.3-15.1 (Tonoki et al. 1991). The two most commonly studied SNPs in the PTH gene are guanine to adenine substitution in intron 2 (*BstBI* polymorphism) and cytosine to adenine substitution in exon 3 (*DraII* polymorphism) (Mullersman et al. 1992). Relationships have been found between the *BstBI* polymorphism and serum total calcium levels (Kanzawa et al. 1999) and bone remodelling (Hosoi et al. 1999). The *DraII* polymorphism was shown to be associated with serum intact PTH level in patients with hyperparathyroidism or hemodialysis (Kanzawa et al. 1999, Gohda et al. 2002), however, not in patients with idiopathic hypoparathyroidism (Goswami et al. 2004). Although the PTH gene has been considered an important candidate gene for osteoporosis, so far only few studies have evaluated the relationship between PTH gene polymorphisms and BMD. Some evidence indicates that both the *BstBI* and *DraII* polymorphisms are associated with BMD (Hosoi et al. 1999, Kanzawa et al. 1999, Katsumata et al. 2002, Deng et al. 2002, Dvornyk et al. 2005). Furthermore, the *BstBI* polymorphism has been suggested to account for 7-9% of the total variances in bone dimensional variables and age-related bone loss in Caucasian females (Gong et al. 1999b). In contrast, few studies have failed to show any associations with BMD (Zhou et al. 2003, Lei et al. 2005). It has also been speculated that PTH gene polymorphisms may act as modifier loci of autosomal dominant hypocalcemia by affecting the penetrance of the activating CaSR mutation (Alvarez-Hernandez et al. 2003).

3 AIMS OF THE STUDY

The main objective of this thesis was to investigate the associations of lactase, VDR, CaSR and PTH gene polymorphisms with calcium homeostasis and bone mineral density in Finns at varying stages of the skeletal life span. The specific aim was to explore gene-diet and gene-gene interactions for the studied gene polymorphisms. This thesis focuses on the four candidate genes for osteoporosis which have a role in calcium homeostasis and bone metabolism, and which imply potential functions that can be measured as biomarkers or intermediate endpoints of calcium homeostasis and bone mineral density. The selection criteria for the polymorphic markers were a replication of the associations found in other populations and a sufficient prevalence of genotypes in the Finnish population. The study designs take into consideration the background and lifestyle factors that may confound the associations between SNPs and bone health. The specific aims of the thesis were

- 1) to study the associations between the C/T₋₁₃₉₁₀ polymorphism of the lactase gene and the consumption of milk and milk products from childhood into young adulthood (unpublished results from the Young Finns Cohort), and peak bone mass and bone mass and density changes in young adulthood (Study I);
- 2) to examine the relationships between the *BsmI* and *FokI* polymorphisms of the VDR gene and calcium and bone metabolism and bone mineral density in adolescents, young adults and postmenopausal women (Studies II, III and IV);
- 3) to investigate the associations of the A986S polymorphism of the CaSR gene and the *BstBI* polymorphism of the PTH gene with calcium homeostasis and bone mineral density in young adults (Study IV);
- 4) to assess dietary interactions in the associations between the lactase and VDR gene polymorphisms and calcium homeostasis and bone mineral density (Studies I, II and IV); and
- 5) to evaluate the role of gene-gene interactions within the VDR, CaSR and PTH gene polymorphisms in calcium homeostasis and bone mineral density in young adults (Study IV).

4 SUBJECTS AND METHODS

4.1 Subjects and study designs

In all studies, anthropometric data (height and weight) were measured during the research visits. All subjects were healthy, and were neither taking medications nor had known conditions affecting bone health. A signed informed consent was obtained from all participants and at the beginning of Study II also from the parents. All study protocols had been approved by the local Ethics Committees.

The subjects of Study I belong to the Cardiovascular Risk in Young Finns Cohort (the Young Finns Cohort) that is an ongoing multi-centre follow-up of atherosclerosis risk factors in young Finns. The subjects were randomly selected from the national population register from the university hospital cities in Finland (Helsinki, Turku, Tampere, Kuopio and Oulu) and the rural municipalities in their vicinity. The first cross-sectional survey was conducted in 1980, and the same subjects were re-examined in follow-up studies in 1983, 1986, 1989, 1992 and 2001 (Raitakari et al. 2008). A subgroup of 671 randomly selected subjects of the original cohort from the five study centres were invited to BMD survey in 1990-1992 (Välimäki et al. 1994), and the same subjects were invited to BMD follow-up measurements in average 12 years later in 2002-2003.

Adolescent subjects for the cross-sectional Study II were enrolled from the upper levels of comprehensive schools in the capital area (Helsinki-Kerava-Porvoo) in 1997. Study III is a cross-sectional study conducted from 1994 to 1995 in Helsinki and comprised of premenopausal and postmenopausal women. The premenopausal women were a mixed group of vegans recruited from the Vegetarian Organization and omnivores were members of the staff at a big clinical laboratory. Postmenopausal women were recruited from the Martha Organisation. They had not received estrogen replacement therapy in the past 5 years, and at least 2 years had passed since their last menstrual cycle. Subjects in the cross-sectional Study IV were a randomly invited group of adults participating in the FINRISK survey organized by the Finnish National Public Health Institute in the Helsinki-Turku-Loimaa area in 1997.

The selected characteristics of the subjects are presented in Table 4.

Table 4. Characteristics of the subjects in Studies I-IV, means (SD).

Study	N	Age (y)	Height (cm)	Weight (kg)	BMI (kg/m ²) / Growth (cm/year)	Genotyping (n)		
I Young Finns Study, whole cohort						Lactase C/T ₋₁₃₉₁₀		
Baseline (1980)	3596	10.4 (5.0)	141.3 (26.0)	38.3 (18.3)	-	-		
females	1832	10.6 (5.0)	139.9 (24.3)	37.4 (16.7)	-	-		
males	1764	10.4 (5.0)	142.6 (27.5)	39.3 (19.8)	-	-		
Follow-up (2001)	2306	31.7 (5.0)	172.1 (9.1)	74.6 (15.9)	25.1 (4.4)	2281		
females	1266	31.7 (5.0)	166.0 (6.0)	67.6 (13.3)	24.5 (4.6)	1256		
males	1040	31.7 (5.0)	179.5 (6.5)	83.1 (14.7)	25.7 (4.1)	1025		
I Young Finns Study, BMD survey						Lactase C/T ₋₁₃₉₁₀		
Baseline (1990-1992)	358	23.8 (3.5)	171.7 (9.0)	66.8 (13.0)	22.5 (3.2)	293		
females	207	23.7 (3.5)	165.9 (5.5)	59.9 (9.7)	21.8 (3.1)	167		
males	151	23.8 (3.4)	179.7 (6.4)	76.1 (10.9)	23.5 (2.9)	126		
Follow-up (2002-2003)	158	36.4 (3.6)	171.8 (8.8)	72.5 (12.8)	24.5 (3.5)	145		
females	91	36.0 (3.6)	166.2 (5.6)	65.8 (10.5)	23.9 (3.9)	83		
males	67	36.7 (3.6)	179.4 (6.1)	81.6 (9.6)	25.3 (2.8)	62		
II Adolescents						VDR (<i>BsmI</i> , <i>FokI</i>)		
all	124	14.7 (0.6)	167.9 (7.7)	56.7 (8.3)	3.6 (3.2)	124		
females	86	14.7 (0.6)	165.3 (5.5)	55.2 (7.9)	2.4 (2.2)	86		
males	38	14.9 (0.7)	174.4 (8.6)	60.5 (8.1)	7.3 (2.9)	38		
III Women						VDR (<i>BsmI</i>)		
all	104	51.9 (12.5)	162.3 (11.9)	66.6 (11.3)	27.1 (22.3)	94		
premenopausal	28	33.4 (7.1)	166.0 (5.4)	60.0 (9.1)	21.8 (3.1)	24		
postmenopausal	76	58.7 (4.5)	160.9 (13.2)	69.0 (11.1)	29.0 (25.8)	70		
IV FINRISK survey						VDR (<i>FokI</i>)	CaSR (A986)	PTH (<i>BstBI</i>)
all	350	37.3 (3.5)	171.1 (8.8)	72.1 (14.5)	24.6 (4.1)	303	286	132
females	215	37.5 (3.4)	165.9 (6.1)	65.5 (12.0)	23.8 (4.3)	189	182	85
males	135	36.9 (3.6)	179.3 (5.6)	82.9 (11.6)	25.9 (3.4)	114	104	47

4.2 Dietary intake and lifestyle

Dietary assessment

In the Young Finns Study, the subjects' daily intake of energy, calcium, lactose and protein were assessed at baseline in 1980 and in follow-ups in 1986 and 2001. The data were obtained with a detailed 48-hour dietary recall assessed in 50% of the subjects at baseline in 1980 and in follow-ups in 1986 and 2001. Dietary interviewers were all trained dietitians and collected information on foods and beverages consumed by subjects during the 2 days prior to the interview. In 1980 and 1986, 3- to 12-year-old children were interviewed together with their mothers, fathers or another accompanying person. As detailed information as possible on the type and amount of foods and drinks consumed was documented on a form by the interviewer. The food composition data used in the 1980s was based on the Finnish food composition tables (maintained by the University of Helsinki) and on analytical data obtained from the local food industry. Foreign food composition tables were used when no appropriate domestic data were available. In 2001, the latest version of the National Food Composition Database was used to calculate the intakes of nutrients (National Public Health Institute, 2003).

At baseline in 1980 and in follow-ups in 1983, 1986, 1989, 1992 and 2001 of the Young Finns Study, longer-term dietary habits and food consumption were assessed with a questionnaire. The questionnaire has been slightly modified over the years to fit the relevant issues according the year and age, but has included the same set of food choices during all follow-ups. In this study, the questionnaire data on the amount of milk consumed (glasses per day) and the frequencies of consumption of cheese, sour milk products (buttermilk, sour whole milk, yoghurt and curd cheese) and ice cream were used as an indicator of the habitual consumption of milk and milk products. The mean of consumption of milk and milk products at follow-ups in childhood and adolescence was calculated to represent the cumulative consumption of milk products. The details of the dietary assessment in the Cardiovascular Risk In Young Finns project have been described previously in studies by Räsänen et al. (1985 and 1991) and Mikkilä et al. (2004).

In BMD survey of Study I and in Studies II - IV, dietary intake of calcium and vitamin D (except for vitamin D at Study I baseline) were estimated by a validated semi-quantitative food frequency questionnaire (FFQ) including pictures of portions sizes and questions about supplements (Lamberg-Allardt et al. 2001). In Studies III and IV, the subjects also

kept four day food records according to instructions given both verbally and in writing, and by using pictures of portions sizes (Haapa et al. 1985). In Study III, a 4-day food record was used for the assessment of calcium intake because it was shown to be a better predictor of the serum PTH concentration than the FFQ method (Kärkkäinen et al. 1998). In Study IV, food records were used for the assessment of phosphorous intake, which is a known confounding factor in calcium homeostasis. During the research visit the FFQs and food records were checked by a nutritionist or a trained research assistant. In Studies II-IV, the dietary intakes of nutrients were calculated using database based on Finnish analyses and international food composition tables (Rastas et al. 1993). In Studies II - IV, S-25OHD concentration was used as a biomarker for vitamin D status, and in Study IV, sodium excretion was used as a biomarker for sodium intake.

Assessment of other lifestyle factors

In Study I, the data on lifestyle variables is based on the questionnaire that was used in all follow-ups (Raitakari et al. 2008). Physical activity was calculated as an index that summarizes the intensity, frequency and duration of leisure-time physical activity as points (Telama et al. 2005). The intensity of physical activity had three options for feeling sweaty and breathless (none, a little, a lot), the frequency six options from never to daily and the duration four options (<20 min, 20-39 min, 40-60 min, >60 min). Smoking behaviour was assessed as the number of cigarettes smoked daily. Consumption of alcoholic beverages was classified as daily, once a week, once a month, less than once a month or never.

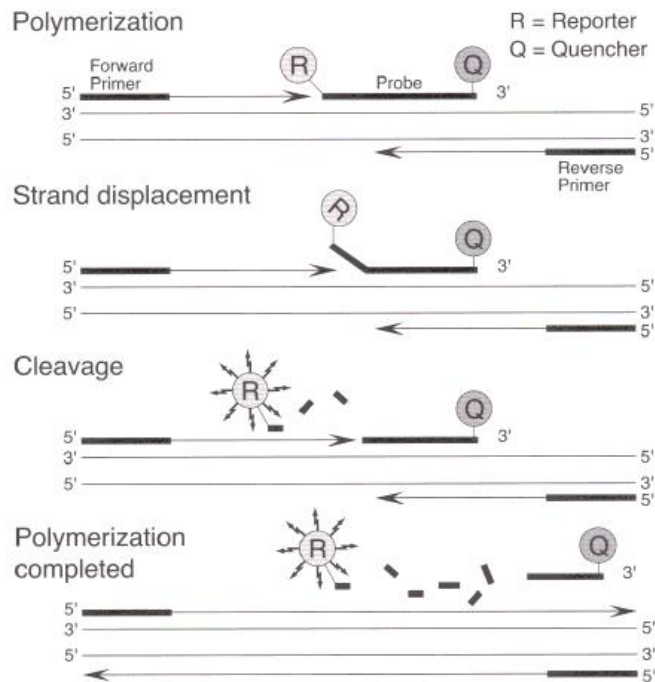
In Studies II-IV, questionnaires were used to collect data on physical activity, smoking habits, and alcohol consumption. Calculation of physical activity varied between Studies II-IV because the questionnaires on life-style factors differed from each other. In Study II, physical activity was calculated based on the minutes per day that were spent with school exercise and leisure activities. In Study III, leisure time physical activity was measured as points, where a weekly period of exercise lasting from half an hour to an hour was equivalent to one point. In Study IV, leisure time physical activity was calculated as minutes per day. In Study II smoking was recorded as the number of cigarettes smoked per month, and in Study III all subjects were non-smokers. In study IV, the number of cigarettes per day and years of smoking were recorded, and Brinkman's index was calculated (cigarettes/day * years of smoking). In Studies II-IV, alcohol consumption was recorded as alcoholic beverages consumed during the two weeks prior to the research visit, and quantified as the total amount of alcohol consumed per day (g/d).

4.3 Laboratory analyses

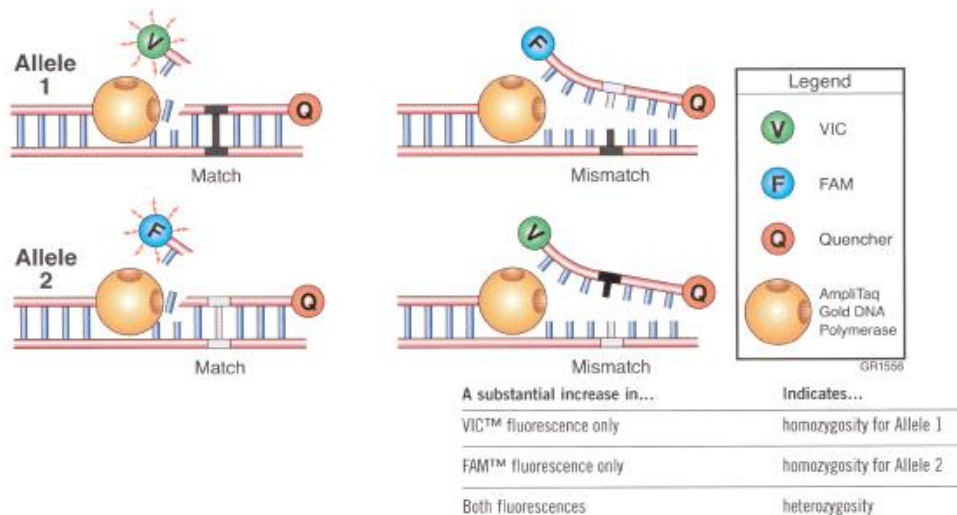
Genotyping

In the Young Finns Study, blood samples for genetic analyses were collected in 2001. DNA was extracted from the peripheral blood leucocytes using a commercially available kit (Qiagen Inc., Hilden, Germany). Lactase C/T-¹³⁹¹⁰ genotyping was performed by employing a 5'-nuclease assay, and fluorogenic allele-specific TaqMan probes and primers (Livak et al. 1995) were used with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA)(Figure 4). Lactase genotyping for the Young Finns Study was performed in the Laboratory of Atherosclerosis Genetics managed by Professor Terho Lehtimäki and located at the Centre for Laboratory Medicine, in the Tampere University Hospital.

In studies II-IV, DNA was isolated from peripheral venous blood by a non-enzymatic extraction method and amplified with a polymerase chain reaction (PCR) method. In Studies II and III, restriction enzymes were used for allele identification and in Study IV, the allele-specific PCR amplification technique was applied. Allele detection was performed by separating PCR products by electrophoresis through agarose gels containing ethidium bromide and visualizing gels under UV-light. Genotyping in Studies II-IV was carried out at the Division of Nutrition, University of Helsinki. Details of genotyping methods in Studies II-IV are presented in Table 5.



A) The main steps of the assay in the polymerase chain reaction (PCR) are polymerization, strand displacement and cleavage. Two dyes, a fluorescent reporter (R) and a quencher (Q), are attached to the probes. During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



B) The allelic discrimination assay is performed under competitive PCR conditions with both probes present; therefore, the mismatched probes are prevented from binding due to stable binding of the exact-match probe. The probes are labelled with a reporter dye and a quencher. When the exact-match probe binds to DNA, the reporter dye fluoresces because it is separated from the quencher. The alleles can be identified by detection of the different fluorescent reporter dyes.

Figure 4 Principles of the SNP screening with allelic discrimination using the 5' nuclease assay (Livak et al. 1995, figures from Applied Biosystems).

Table 5. Genotyping methods in Studies II -IV.

Polymorphism	VDR <i>BsmI</i>	VDR <i>FokI</i>	CaSR A986S	PTH <i>BstBI</i>
Studies in which used	II, III, IV	II, IV	IV	IV
Primers	<i>forward</i> 5'CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA3' <i>reverse</i> 5'AAC CAG CGG GAA GAG GTC AAG GG3'	<i>forward</i> 5'ATG GAA ACA CCT TGC TTC TTC TCC CTC3' <i>reverse</i> 5'AGC TGG CCC TGG CAC TGA CTC TGC TCT3'	<i>forward1</i> 5'GCT TTG ATG AGC CTC AGA AGA TCG3' <i>forward2</i> 5'AGG GTC ACC TTC TCA CTG ACG TTT GAT GAG CCT CAG AAG TAC T3' <i>reverse</i> 5'CTC TTC AGG GTC CTC CAC CTC T3'	<i>forward</i> 5'CAT TCT GTG TAC TAT AGT TTG3' <i>reverse</i> 5'GAG CTT TGA ATT AGC AGC ATG3'
PCR cycle				
reaction volume	50 µl	25 µl	20 µl	25 µl
number of cycles	32	30	30	36
denaturation	93°C, 1 min	94°C, 30 s	94°C, 15 s	94°C, 30 s
annealing	62°C, 1 min	60°C, 30 s	57°C, 30 s	53°C, 30 s
extension	72°C, 45 s	72°C, 30 s	72°C, 30 s	72°C, 1 min
Restriction enzyme incubation	<i>BsmI</i> 7.5U/sample 65°C, 3 h	<i>FokI</i> 3.2U/sample 37°C, 2 h	-	<i>BstBI</i> 5U/sample 65 °C, 3 h
Reference	Morrison et al. 1994	Gross et al. 1996	Cole et al. 2001	Mullersman et al. 1992

Measurements of calcium and bone metabolism

In Studies II-IV, fasting overnight blood samples were collected during the study visit. Serum was separated from blood samples by centrifuging except in the competitive protein binding assay (Study III) serum was extracted with acetonitrile. In Studies III and IV, blood samples were collected in winter when sunlight exposure in Finland is not sufficient for skin synthesis of vitamin D precursors. In Study IV, 24-hour urine samples were collected on three separate days before the study visit. All serum and urine samples were stored at -20°C until analysed.

In Study IV, the serum ionized calcium concentration was measured within 90 min after blood sampling with a selective ion analyzer (ISE Ca⁺⁺/pH Analyzer 634; Ciba Corning Diagnostics, Halstead, United Kingdom) and adjusted to pH 7.4. The measurements of serum phosphorous (S-P), urine calcium (U-Ca), urine sodium (U-Na) and creatinine were done with an automated Kone-Lab spectrophotometer (Thermo Clinical Labsystems Ltd, Espoo, Finland) using routine laboratory methods. The intra- and inter-assay CV for these analyses were less than 7.5%. In Study IV, the average of three days' urine collections was used to calculate U-Ca and U-Na, and the concentrations were corrected for creatinine excretion. All measurements of calcium and bone metabolism in Studies II-IV were carried out at the Division of Nutrition, University of Helsinki.

In Study III, an oral strontium absorption test with non-radioactive stable strontium chloride, which reflects calcium absorption capacity (Milson et al. 1987), was performed on a subgroup of 79 volunteer subjects. Two and half mmol of strontium chloride was given in water with a light standardised meal in the morning. A blood sample was taken four hours after administration of the strontium. Serum strontium concentration was measured by atomic absorption spectrometry (Perkin-Elmer 1100B, Norwalk, Connecticut, USA) at 460.7 nm using a lanthanum chloride/hydrochloric acid diluent to remove interference with phosphate. The fractional calcium absorption was calculated using the equation: $(0.15 \times \text{strontium concentration} \times \text{weight}) / 25$. The CV% was 1.8 at the level of 20 µmol/l.

Summary of the biochemical methods for measuring calcium and bone metabolism in Studies II-IV are presented in Table 6.

Table 6. Biochemical measurements of calcium and bone metabolism in Studies II-IV.

	Study	Method	Manufacturer/ Reference	Reference range	CV% intra	CV% inter
S-iCa	IV	Ion selective analyser	Ciba Corning Diagnostics	1.18-1.30 mmol/l	-	-
S-iPTH	III, IV	IRMA	Nichols	10-65 ng/l	1	4
S-25OHD	II, III, IV	Study III: CPBA	In house method ¹	25-120 nmol/l	15	14
		Studies IV and V: RIA	Incstar	25-120 nmol/l	10	15
BALB	III	Enzyme immunoassay	Metra	premenopausal women 11.6-29.6 U/L postmenopausal women 14.2-42.7 U/L men 15-41.3 U/L	4	6
Osteocalcin	III	IRMA	Nichols	women 0.2-1.8 nmol/l men 0.4-2.4 nmol/l	8	4
PICP	III	RIA	Orion	women 50-170 µg/l men 38-202 µg/l	2	3
ICTP	III	RIA	Orion	women 1.6-5.3 ug/l men 1.4-5.2 µg/l	4	4

Abbreviations: S-iCa = serum ionized calcium; S-iPTH = serum intact parathyroid hormone; S-25OHD = serum 25-hydroxy-vitamin-D; BALP = bone-specific alkaline phosphatase; PICP = carboxyterminal propeptide of collagen type I; ICTP = cross-linked telopeptide of type I collagen; CPBA = competitive protein binding; IRMA = immunoradiometric assay; RIA = radioimmunoassay.

¹Törnquist and Lamberg-Allardt 1987.

4.4 Bone mineral density measurements

In Study I, areal BMD at axial bone sites was measured in five different study centres. The densitometers used in the study centres were as follows: in Helsinki a Hologic QDR-1000 densitometer (Hologic Incorporation, Bedford, MA, USA, lumbar spine *in vivo* CV% 1.0), in Oulu and Kuopio, Lunar DPX densitometers (GE Healthcare, Waukesha, WI, USA, lumbar spine *in vivo* CV% 0.8 and 0.8, respectively), and in Turku and Tampere, Norland XR-26 densitometers (Norland Corporation, Fort Atkinson, WI, USA, lumbar spine *in vivo* CV% 0.7 for both densitometers). At baseline, the BMD values obtained from the Hologic and Lunar densitometers were adjusted to Norland values by measuring nine healthy women with all five densitometers used (details in Valimaki et al. 1994). The baseline and follow-up measurements were performed using the same densitometry unit. The drifts in the measurement levels between the baseline and follow-up were checked using the lumbar spine phantom results obtained from the densitometer units. In Kuopio, where the Lunar DPX densitometer had been replaced by a Lunar Prodigy densitometer, the follow-up measurement level was adjusted to the baseline level using regression equations. Detailed information regarding the regression equation used for the adjustment and the reproducibility of the densitometer measurements is given in Publication I.

In Study II, areal BMD at the distal forearm was measured with a DTX-200 Osteometer (Osteometer MediTech A/S, Denmark, CV% 0.7). In Study III, areal BMD at the axial bone sites was measured with DXA (Lunar Radiation Corp, Madison, WI, USA), and subjects diagnosed for a collapsed vertebrae by a physician were excluded from the statistical analysis where lumbar spine BMD was used as a dependent variable. In Study IV, areal BMD at the distal forearm was measured by a portable DXA-densitometer (PIXI 1.34, Lunar Corporation, MA, USA, CV% 0.4). In Studies II and IV, quantitative ultrasound measurements (QUS; Sahara, Hologic Inc. Waltham, MA, USA, CV% 2.3) at the calcaneus were used to determine broadband ultrasound attenuation (BUA, db/MHz) and ultrasound transit velocity (SOS, m/s).

Bone density measurements in Studies I-IV are summarized in Figure 5.

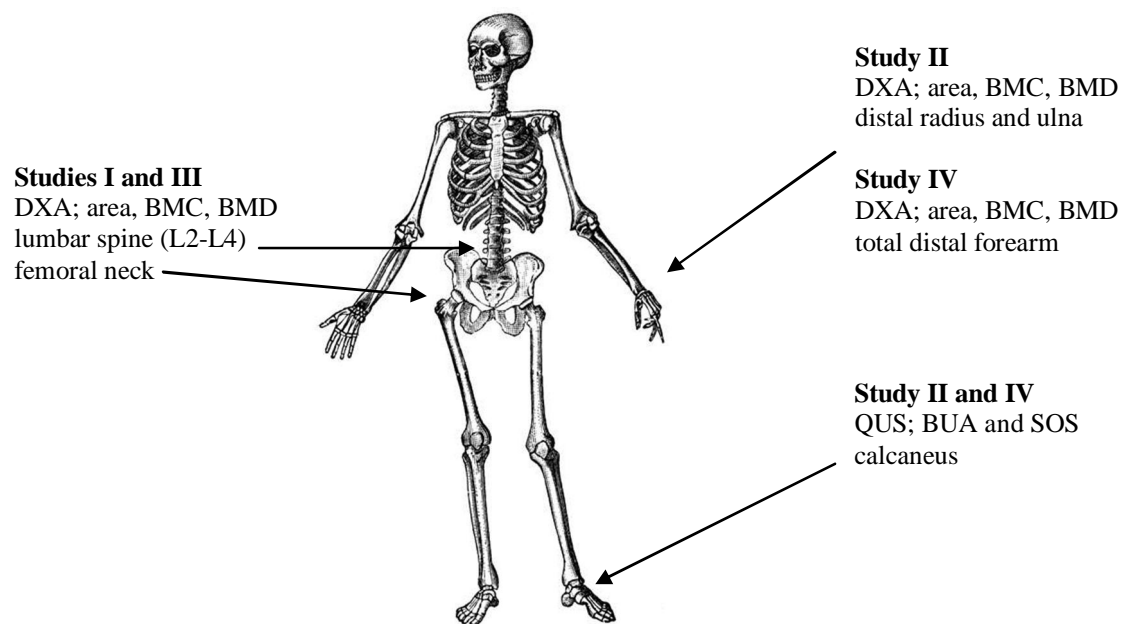


Figure 5 Bone density measurement sites in Studies I-IV.

4.5 Statistical methods

The data are expressed using means and either standard deviations (SD), standard error of mean (SEM) or 95% confidence intervals (95% CI) for distributions. After testing for normality with the Shapiro-Wilk's test, logarithmic transformations were used if necessary. Levene's statistics were used to test the homogeneity of variances across the lactase, VDR, CaSR, and PTH genotypes.

The deviations from expected allele frequencies (Hardy-Weinberg equilibrium, HWE) were tested for all the SNPs studied using the GENEPOP program (<http://genepop.curtin.edu.au>) (Raymond and Rousset 1995). Hardy-Weinberg proportions represent the binomial distribution of alleles that is seen in a population and remains constant when there are no external pressures such as non-random mating, mutations, selection, limited population size, random genetic drift and gene flow. The HWE probability-test (exact HW test) evaluates whether the allele frequency differs from the expected proportions: p^2 , $2p(1-p)$, and $(1-p)^2$, where p is the minor allele frequency. When the alternative hypothesis of interest was heterozygote excess or deficiency, a more powerful test, the score test (U test), was applied (see Raymond and Rousset 1995).

The differences in the dependent variables between the lactase, VDR, CaSR and PTH genotypes were compared with analysis of variance (ANOVA) and covariance (ANCOVA). The lifestyle factors that were used as confounders in ANCOVA are summarized in Table 7. For the variables with unequal variances, Kruskal-Wallis non-parametric analysis was applied. In Studies II and IV, some analyses were performed sexes pooled. If sexes were pooled, two-way ANCOVA was applied using sex and genotype as independent variables. Fisher's least significant difference method (Fisher's LSD) was applied for the pairwise comparisons of the genotypes when ANOVA p-values were below 0.05; otherwise, i.e. when p-values exceeded 0.05, genotypes were compared with Bonferroni (Studies I, III and IV) or Scheffe (Study II) post hoc tests. If the Levene's test results were significant, the non-parametric post hoc Games-Howell test was used.

In the Young Finns Study, the changes in milk consumption over a subject's childhood years were tested with repeated measures analysis of variance (RANOVA). Pearson's chi-square (χ^2) test was applied to compare categorical variables between the lactase genotypes. In Study I, the determinants of relative changes in bone area, BMC and BMD at both measurement sites were analysed with multiple regression analysis by entering the

following variables simultaneously into the model: birth year, weight change, and changes in calcium intake, physical activity, smoking and alcohol consumption.

In Study III, statistical analyses were carried out with BMDP statistical software in a VAX/VMS minicomputer system (Digital Equipment Corporation). Since the BMDP program was no longer available in the Helsinki University network, in Studies I, II and IV, statistical analyses were carried out with the Statistical Package for Social Sciences (SPSS) and versions 10, 12 and 15 for Windows were used (SPSS Inc., Chicago, IL, USA). P-values below 0.05 were considered statistically significant.

Table 7. Lifestyle confounders included in the statistical analyses in Studies I-IV. See chapter 4.2 for details of the assessment of lifestyle factors.

	Dietary intake	Physical activity	Smoking	Alcohol consumption
Study I	Calcium intake	Leisure time physical activity index as points ³	Cigarettes/day	Frequency of alcohol consumption
Study II	Calcium intake S-25OHD ¹	School exercise and leisure activities as min/d	Cigarettes/month	-
Study III	Calcium intake S-25OHD ¹	Leisure time physical activity as points ⁴	- (Subjects were non-smokers)	-
Study IV	Calcium intake Sodium intake (U-Na) ² S-P S-25OHD ¹	Leisure time physical activity as min/d	Brinkman's index ⁵	Alcohol consumption g/d

¹ S-25OHD is the indicator for vitamin D status.

² Urinary sodium excretion is the best indicator of habitual sodium intake.

³ Physical activity index that summarizes the intensity, frequency and duration of leisure-time physical activity as points.

⁴ A weekly period of exercise lasting from half an hour to an hour was equivalent to one point.

⁵ Brinkman's index = smoking calculated as cigarettes/d * smoking years.

5 RESULTS

5.1 Genotype distributions of the studied SNPs (Studies I-IV)

The distributions of genotypes are presented in Table 8. In pre- and postmenopausal women (Study III), the genotype distribution for the *BsmI* polymorphism of the VDR gene differed from the Hardy-Weinberg equilibrium with a significant heterozygote deficiency (U-test; $p=0.020$).

Table 8. Genotype distributions (%) and exact tests for Hardy-Weinberg equilibrium in Studies I-IV.

Lactase C/T _{.13910}					Lactase C/T _{.13910}				
<i>HWE</i> ¹					<i>HWE</i> ¹				
	T/T	C/T	C/C	<i>p-value</i>		T/T	C/T	C/C	<i>p-value</i>
Study I					Study I				
n = 2281	33	49	18	0.813	n = 293	33	49	18	0.329
VDR <i>BsmI</i>					VDR <i>FokI</i>				
	BB	Bb	bb			FF	Ff	ff	
Study II					Study II				
n = 86	18	42	40	0.192	n = 86	40	43	17	0.432
Study III					Study III				
n = 93	16	34.5	49.5	0.035		-	-	-	-
Study IV					Study IV				
	-	-	-	-	n = 303	42	46	12	1.0
CaSR A986S					PTH <i>BstBI</i>				
	AA	AS	SS			BB	Bb	bb	
Study IV					Study IV				
n = 286	79	19	2	0.248	n = 132	16	42	42	0.265

¹ The exact test for Hardy-Weinberg equilibrium

5.2 The lactase gene C/T₋₁₃₉₁₀ polymorphism, consumption of milk products, and calcium and lactose intakes (Young Finns Study, unpublished results)

From childhood, those with the C/C₋₁₃₉₁₀ genotype consumed less milk than did those with the other lactase genotypes, but the consumption of cheese or sour milk products did not differ between the lactase genotypes ($p < 0.02$ for 6-, 12- and 18-year-old and adult females, and 9-, 12- and 15-year-old and adult males). The proportions of those who reported not drinking milk were highest in the C/C₋₁₃₉₁₀ genotype at all age points in both sexes except in 9-year old males (Figure 6A). The avoidance of milk drinking increased with age in both sexes but did not differ between the lactase genotypes (RANOVA p -values for milk consumption*lactase genotype were 0.42 for females and 0.51 for males).

In females, there was a trend toward lower daily calcium and lactose intakes for the C/C₋₁₃₉₁₀ genotype from 9 years of age (ANOVA $p = 0.025$, C/C vs. T/T $p = 0.06$ and C/C vs. C/T $p = 0.007$), whereas in males, the genotypes did not differ in lactose intake before the age of 18. In adulthood, the C/C₋₁₃₉₁₀ genotype was associated with lower daily calcium and lactose intakes in both sexes, but the difference in calcium intake was only marginally significant (Table 9). In both sexes, the lactase genotypes differed significantly in intakes of calcium, lactose, protein and vitamin D from milk but not from other milk products. In females, the proportions of those with inadequate calcium intake were highest in the C/C₋₁₃₉₁₀ genotype at 9 and 15 years of age and in adulthood (Figure 6B). In males, the C/C₋₁₃₉₁₀ genotype was more susceptible to inadequate calcium intake than other genotypes only in adulthood.

In adulthood, low-lactose or milk-free diets were more common in those with the C/C₋₁₃₉₁₀ genotype in comparison to the other lactase genotypes: in females 6.5%, 6.4% and 22.4% for T/T, C/T and C/C genotypes, respectively ($\chi^2 p < 0.001$) and in males 5.4%, 5.7% and 12.3% for T/T, C/T and C/C genotypes, respectively ($\chi^2 p = 0.009$). In females, the lactose content of milk products consumed was lowest in the C/C₋₁₃₉₁₀ genotype (Table 9).

Adult females with the C/C₋₁₃₉₁₀ genotype who consumed milk and milk products with a higher lactose content had lower calcium intake than those in the other lactase genotypes ($p_{\text{interaction}} = 0.022$), whereas in lower lactose content tertiles calcium intake did not differ between the lactase genotypes (Figure 7). In males, the trend toward differences between the lactase genotypes was similar to that for females but was not significant ($p_{\text{interaction}} = 0.136$).

Table 9. Dietary intakes of selected nutrients (48-hour dietary recall) in adulthood (24-39 y) by lactase genotype. Data expressed as mean and SD.

	Females							Males						
	T/T n = 193 (34%)		C/T n = 273 (48%)		C/C n = 100 (18%)		p-value ¹	T/T n = 152 (33%)		C/T n = 232 (50%)		C/C n = 81 (17%)		p-value ¹
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD	
Calcium intake from all sources (mg/d)	1 160	470	1 174	479	1 048	462	0.07	1 413	727	1 497	661	1 313	563	0.09
Milk products as sources of calcium	886	468	889	466	766	447	0.06	1 100	696	1 183	634	992	537	0.06
milk	332	321	361	300	215	229	< 0.001 ²	523	467	553	517	351	305	0.009 ²
cheese	382	306	371	322	376	326	0.93	438	412	476	376	524	397	0.28
sour milk products	143	156	128	162	149	178	0.44	111	176	124	220	92	141	0.41
Lactose intake from all sources (g/d)	18.8	13.2	19.3	13.3	13.1	9.8	< 0.001 ²	26.0	19.9	28.1	21.5	18.7	13.1	0.003 ²
Milk products as sources of lactose	17.6	13.2	18.3	13.1	12.0	9.5	< 0.001 ²	24.7	19.8	26.5	21.3	17.3	12.7	0.003 ²
milk	13.3	23.2	14.4	12.6	8.0	8.92	< 0.001 ²	21.3	19.1	22.7	21.2	14.4	13.0	0.006 ²
cheese	0.20	0.44	0.19	0.54	0.17	0.38	0.86	0.20	0.49	0.12	0.31	0.14	0.38	0.13
sour milk products	3.15	3.87	2.65	3.71	2.91	3.95	0.38	2.13	3.33	2.65	5.21	1.86	2.90	0.29
Lactose content of milk products (g/100 g) ³	3.44	1.09	3.60	1.03	3.16	1.15	0.003	3.72	1.03	3.74	1.06	3.44	1.14	0.09
Protein intake from all sources (g/d)	68.9	19.8	68.9	27.1	66.3	19.2	0.05	96.0	35.0	102.9	34.3	98.3	26.1	0.13
Milk products as sources of protein	24.7	13.2	24.9	12.9	21.2	12.0	0.012	30.6	19.0	32.5	17.4	28.2	15.7	0.15
milk	8.69	8.39	9.42	7.79	5.59	5.92	< 0.001 ²	13.7	12.1	14.5	13.5	9.2	8.03	0.010 ²
cheese	11.7	9.54	11.5	9.87	11.3	9.06	0.94	13.4	11.8	14.2	10.9	15.9	12.3	0.28
sour milk products	3.60	3.83	3.24	3.72	3.72	4.47	0.47	2.78	4.46	3.15	5.58	2.38	3.62	0.45
Vitamin D intake from all sources (µg/d)	3.69	3.48	4.94	19.3	3.30	2.58	0.47	5.61	6.91	4.76	4.49	4.20	3.15	0.86 ²
Milk products as sources of vitamin D	0.35	0.23	0.38	0.24	0.30	0.20	0.043	0.49	0.38	0.51	0.39	0.34	0.23	0.005 ²
milk	0.19	0.21	0.21	0.22	0.13	0.17	< 0.001 ²	0.31	0.35	0.32	0.38	0.17	0.18	0.003 ²
cheese	0.09	0.08	0.09	0.08	0.09	0.08	0.90	0.11	0.10	0.11	0.09	0.13	0.10	0.29
sour milk products	0.05	0.07	0.05	0.09	0.06	0.09	0.59	0.04	0.10	0.05	0.11	0.02	0.05	0.20

¹ One-way analysis of variance; ² Kruskal-Wallis non-parametric test; ³ The lactose content of milk products was calculated as grams of lactose per 100 grams of milk and milk products consumed.

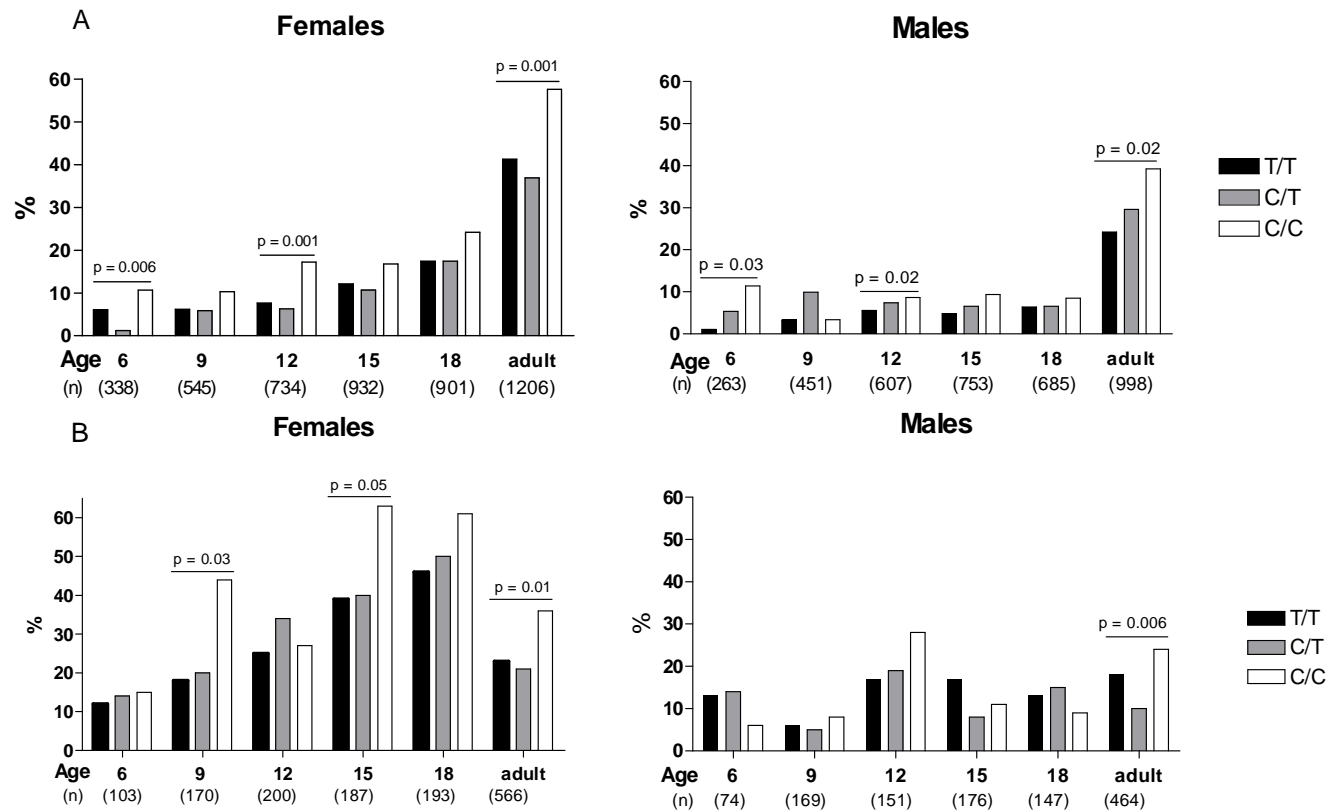


Figure 6 Proportions (%) of those (A) not drinking milk and those with (B) calcium intake lower than recommended, by lactase genotypes in the Young Finns Study (unpublished results). Proportions of those who do not drink milk were assessed with a questionnaire on habitual food choices. Lower than recommended calcium intake was determined by the Finnish Nutritional Recommendations (2005) for calcium intake: children 6-9 y: 700 mg/d; children and adolescents 10-20 y: 900 mg/d; and adults: 800 mg/d. P-values are from the χ^2 -test.

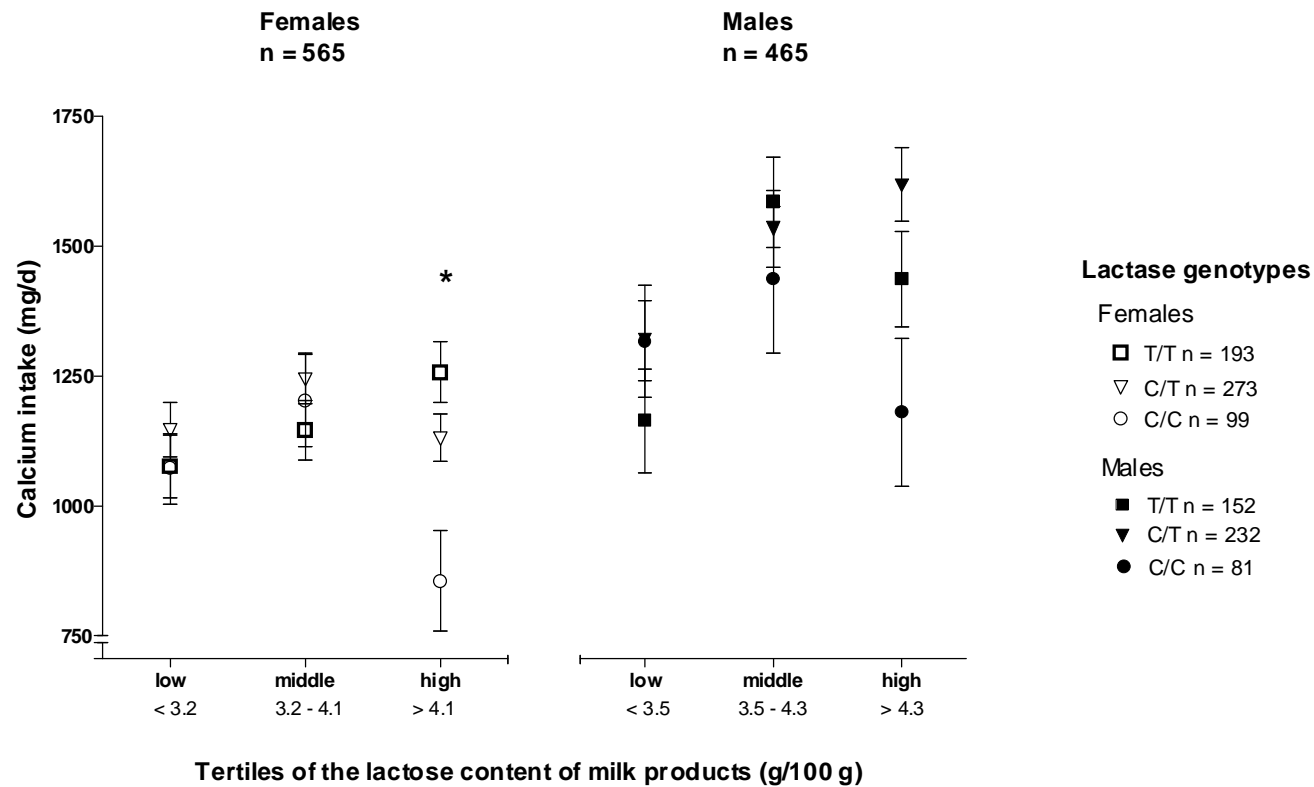


Figure 7 Calcium intake in tertiles of the lactose content of milk and milk products consumed in adulthood (age range 24-39 y) by lactase genotypes in the Young Finns Study (unpublished results). Data is expressed as mean and SE. Data on calcium intake and milk consumption was assessed by 48-hour dietary recall. The lactose content of milk products was calculated as grams of lactose per 100 grams of milk and milk products consumed. In females, two-way ANOVA for lactase genotype $p = 0.064$ and for interaction genotype*lactose content of milk products consumed $p = 0.022$. In males, two-way ANOVA for lactase genotype $p = 0.092$ and for interaction genotype*lactose content of milk products consumed $p = 0.136$.

5.3 The lactase gene C/T₋₁₃₉₁₀ polymorphism and BMC and BMD changes in young adulthood (Study I)

At baseline, PBM did not differ between lactase genotypes according to either bone site or sex when using age, weight, smoking, alcohol consumption and physical activity as covariates. During the 12-year follow-up, the average calcium intake increased by 89 mg/d in females and decreased by 310 mg/d in males (t-test for sex difference $p < 0.001$). The relative changes in both lumbar spine and femoral neck BMC and BMD differed between the sexes (Table 10). In females, the lumbar spine BMC and BMD and the femoral neck BMC increased, whereas the femoral neck BMD decreased. In males, BMC and BMD decreased at both bone sites.

In both sexes, the youngest age cohort had the greatest decrease in femoral neck BMC and BMD; however, in males the difference in BMD changes was not significant (Publication I, Table 3). Increased calcium intake predicted an increase in femoral neck BMD in both sexes ($B = 0.007 \text{ g/cm}^2/\text{mg}$, $p = 0.002$ in females; $B = 0.006 \text{ g/cm}^2/\text{mg}$, $p = 0.045$ in males). In females, physical activity was associated with increased lumbar spine BMD in females ($B = 0.091 \text{ g/cm}^2/\text{activity point}$, $p = 0.023$). Other variables in the regression models did not correlate with BMC or BMD changes.

After adjusted for age, weight change (%), and changes in alcohol consumption, smoking and physical activity, females with the C/C₋₁₃₉₁₀ genotype had the greatest increase in femoral neck bone area and males with the T/T₋₁₃₉₁₀ genotype in lumbar spine bone area (Publication I, Table 4). In males, the C/C₋₁₃₉₁₀ genotype had the greatest BMC and BMD losses at the lumbar spine and BMC loss at the femoral neck (Table 10). However, the differences were borderline significant only in the lumbar spine BMD, whereas the other adjusted differences in BMC or BMD changes were not significant in either sex.

Table 10. Relative changes in bone area, mineral content and density during the 12-year follow-up in adulthood by lactase genotypes (Study I).

	Females		Males		<i>p for sex (crude)</i>
	Crude	Adjusted ¹	Crude	Adjusted ¹	
Lumbar spine area	↑		↑		<i>0.220</i>
T/T	↑	↓	↑	↑	
C/T	↑	↑↑	↓	↓	
C/C	↑↑	↑	↑↑	↑↑	
<i>p for genotype</i>	<i>0.217</i>	<i>0.084</i>	<i>0.233</i>	<i>0.048</i>	
Lumbar spine BMC	↑		↓		<i>0.003</i>
T/T	↑	↑	↑	↑	
C/T	↑↑	↑↑	↓	↓	
C/C	↑	↑	↓↓	↓↓	
<i>p for genotype</i>	<i>0.388</i>	<i>0.375</i>	<i>0.124</i>	<i>0.114</i>	
Lumbar spine BMD	↑		↓		<i>0.001</i>
T/T	↑	↑↑	↑	↑	
C/T	↑↑	↑	↓	↓	
C/C	↑	↓	↓↓	↓↓	
<i>p for genotype</i>	<i>0.725</i>	<i>0.343</i>	<i>0.180</i>	<i>0.091</i>	
Femoral neck area	↑		↑		<i>0.154</i>
T/T	↑	↑	↑↑	↑	
C/T	↑	↑	0	↑	
C/C	↑↑	↑↑	↑	↓↓	
<i>p for genotype</i>	<i>0.318</i>	<i>0.055</i>	<i>0.521</i>	<i>0.326</i>	
Femoral neck BMC	↑		↓		<i>0.005</i>
T/T	↓	↓	↓↓	↓	
C/T	↑	↑	↓	↓	
C/C	↑↑	↑↑	↓	↓↓	
<i>p for genotype</i>	<i>0.167</i>	<i>0.565</i>	<i>0.878</i>	<i>0.651</i>	
Femoral neck BMD	↓		↓		<i>0.001</i>
T/T	↓↓	↓↓	↓↓	↓	
C/T	↓	↓	↓	↓↓	
C/C	↑	↓	↓	↓	
<i>p for genotype</i>	<i>0.189</i>	<i>0.968</i>	<i>0.822</i>	<i>0.693</i>	

¹ Adjusted for age, weight change (%), and changes in alcohol consumption, smoking and physical activity.
↑ increase; ↓ decrease; ↑↑ greatest increase between the lactase genotypes; ↓↓ greatest decrease between the lactase genotypes.

When the lumbar spine BMD changes during the follow-up were compared with the densitometer-specific least significant change (LSC), in females, bone gain was more common than bone loss, whereas in males bone loss dominated (χ^2 -test $p = 0.022$)(Figure 8). There were no differences in LSCs between the lactase genotypes in either sex.

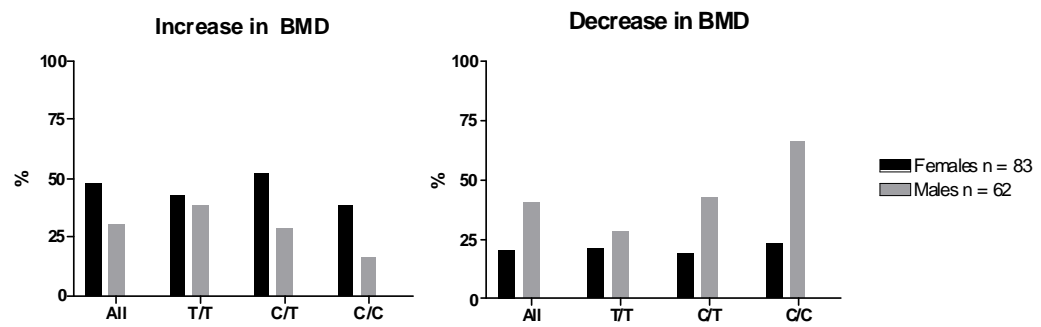


Figure 8 Proportions (%) of those with an increase or a decrease in the lumbar spine BMD during the 12-year follow-up (Study I). Sex-difference in lumbar spine bone loss, χ^2 -test $p=0.022$.

5.4 The VDR gene *BsmI* and *FokI* polymorphisms, calcium homeostasis and BMD (Studies II-IV)

In adolescents (Study II), the *BB* genotype was associated with the highest BMC at the distal radius and ulna, when adjusted for sex, weight, state of growth, lifestyle, *FokI* genotype and *BsmI**sex interaction (ANCOVA $p=0.07$ for BMC at radius and $p=0.024$ for BMC at ulna)(Figure 9A). Furthermore, the *Ff* genotype was associated with the highest calcaneal BUA and SOS values (ANCOVA $p=0.08$ for SOS and $p=0.021$ for BUA)(Figure 9B). When girls and boys were analysed separately, the *FokI* genotypes differed significantly only in boys.

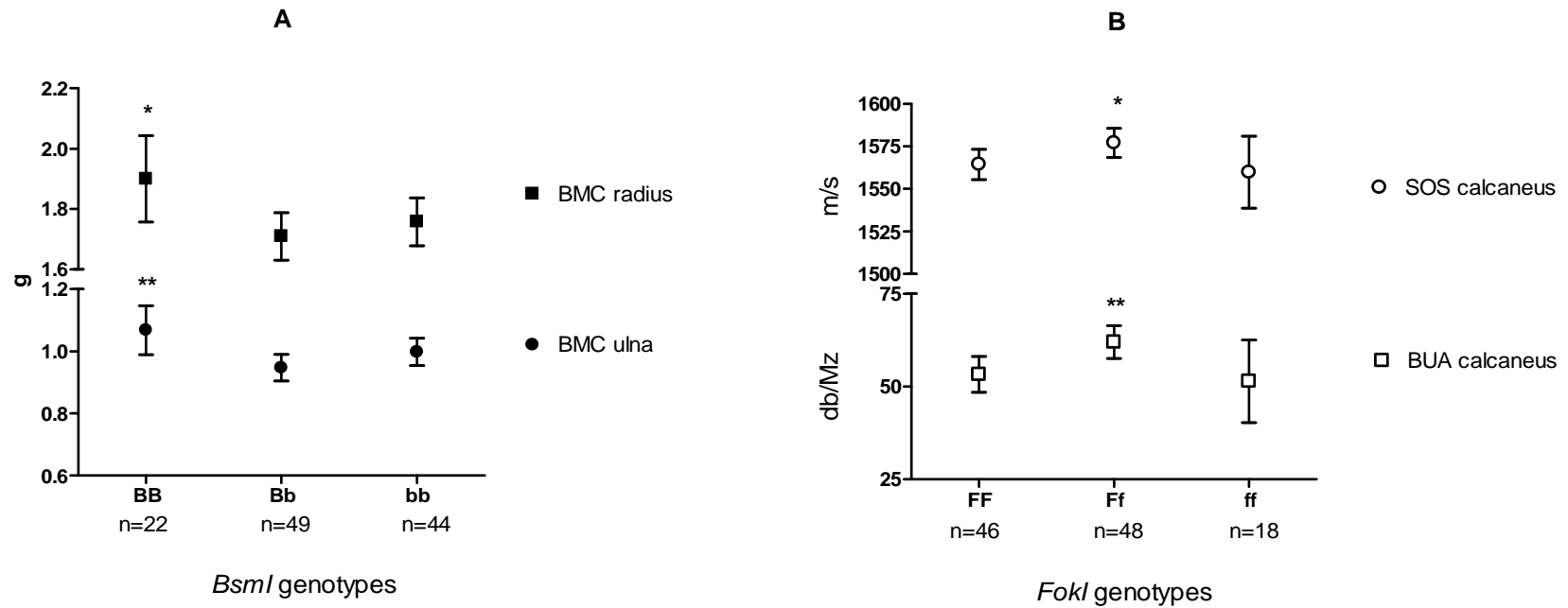


Figure 9 **A.** Bone mineral content (BMC) at the distal radius and ulna in adolescent boys and girls according to *BsmI* genotype (Study II). ANCOVA $p=0.07$ for BMC at the radius and $p=0.024$ for BMC at the ulna using weight, ratio of forearm length to height, calcium intake, physical activity, smoking, S-25OHD, sex, *FokI* genotype and *BsmI* genotype*sex interactions as covariates. In pairwise comparisons *BB* vs. *Bb*: * $p=0.066$ (Bonferroni) and ** $p=0.009$ (LSD). **B.** Broadband ultrasound attenuation (BUA) and speed of sound (SOS) at the calcaneus in adolescent boys and girls according to *FokI* genotype (Study II). ANCOVA $p=0.08$ for SOS and $p=0.021$ for BUA using weight, ratio of forearm length to height, calcium intake, physical activity, smoking, S-25OHD, sex, *BsmI* genotype and *FokI* genotype*sex interactions as covariates. In pairwise comparisons *Ff* vs. *FF*: * $p=0.04$ and ** $p=0.01$ (LSD).

In pre- and postmenopausal women (Study III), there were no differences in the serum concentrations of iPTH, PICP, ICTP, BALP and osteocalcin or in the intestinal strontium absorption between the *BsmI* genotypes. The S-25OHD concentration was highest for the *BB* genotype (28.1, 22.5 and 22.5 $\mu\text{g/l}$ for the genotypes *BB*, *Bb* and *bb*, respectively) when both pre- and postmenopausal women were pooled and the analysis was adjusted for age, BMI, calcium and vitamin D intake (ANCOVA $p=0.02$; LSD *BB* vs. *Bb* $p=0.01$ and *BB* vs. *bb* $p=0.009$). The *BB* genotype had the highest lumbar spine BMD (ANCOVA $p=0.07$) when adjusted for age, BMI, calcium intake, S-25OHD, and physical activity.

In young adults (Study IV), the *FokI* genotypes did not differ in S-iCa, S-P, S-PTH or U-Ca concentrations in either sex (Publication IV, Tables 3-5). Furthermore, none of the forearm BMD or calcaneal ultrasound variables differed between the *FokI* genotypes.

5.5 The CaSR gene A986S and PTH gene *BstBI* polymorphisms, calcium homeostasis and BMD (Study IV)

When sexes were pooled, the S-iCa concentrations were higher in the *AS* and *SS* genotypes of the CaSR A986S polymorphism than in the *AA* genotype (LSD *AA* vs. *AS*, $p = 0.002$; *AA* vs. *SS*, $p = 0.003$; adjusted for BMI, S-25OHD and calcium intake, sex and sex*A986S genotype interaction)(Publication IV, Table 5). When females and males were analysed separately, the *AA* genotype differed from the combined group of the *AS* and *SS* genotypes in both sexes (LSD *AA* vs. *AS/SS* $p = 0.001$ and $p = 0.02$ in females and males, respectively). The *BstBI* polymorphism of the PTH gene was not associated with differences in S-iCa concentrations, and the S-PTH concentrations and urinary calcium excretion did not differ significantly between the CaSR A986S or the *BstBI* genotypes in either sex and (Publication IV, Tables 3-5). Although not significant, in females, there was a consistent decline of 1.4 mmol/l in the U-Ca concentrations between the *BB*, *Bb* and *bb* genotypes respectively (Publication IV, Table 3).

The forearm BMD and the calcaneal ultrasound variables did not differ between the CaSR A986S genotypes in either sex or when sexes were pooled (Publication IV, Tables 3-5). The forearm BMD was the lowest for the *BstBI* genotype *bb* in both sexes (LSD in females: *bb* vs. *Bb* $p=0.01$ and *bb* vs. *BB* $p=0.014$, and in males: *bb* vs. *Bb* $p=0.01$) (Publication IV, Tables 3 and 4). The differences between the *BstBI* genotypes were also similar for the calcaneal ultrasound measurements, but only BUA values differed significantly when sexes were pooled (LSD *bb* vs. *Bb* $p=0.067$ and *bb* vs. *BB* $p=0.015$)(Publication IV, Table 5).

The associations of the CaSR and PTH gene polymorphisms with calcium homeostasis and BMD in young adults (Study IV) are summarized in Figure 10.

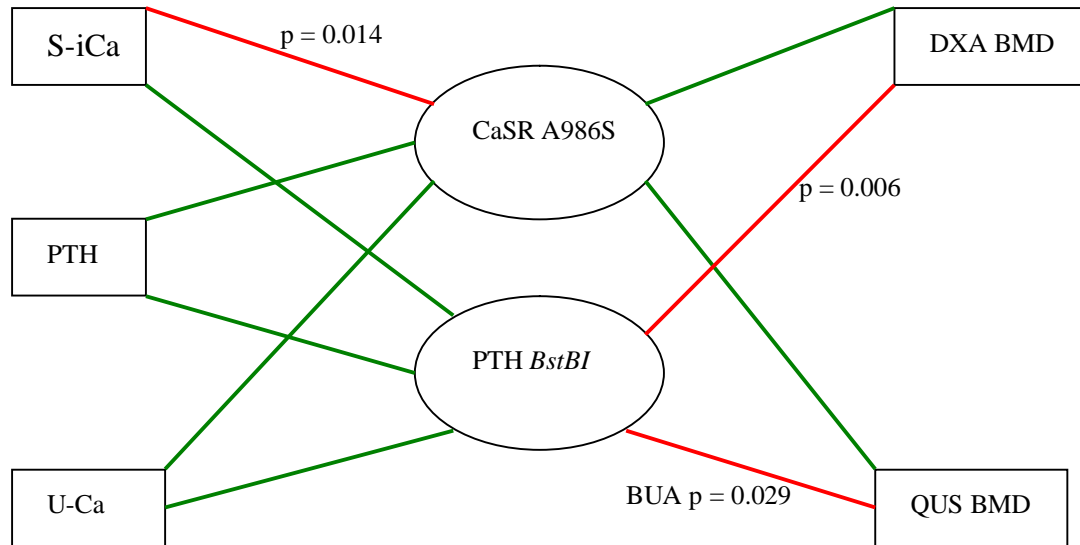


Figure 10 Summary of the associations between the CaSR and PTH gene polymorphisms and calcium homeostasis and BMD (Study IV, sexes pooled). Green color represents an insignificant association and red color a significant association.

5.6 Diet-gene interactions (Studies I, II, IV)

In the Young Finns Study (Study I), the interaction effect between the tertiles of cumulative milk product consumption from childhood to adulthood and lactase genotypes on PBM was tested in females and males separately. When the analyses were adjusted for birth year and body weight, none of the interactions were significant. In the BMD follow-up survey, interactions between changes in calcium intake and lactase genotypes showed no significant effects on bone mass changes in either sex.

Study II in adolescents revealed no interactions between calcium intake and the *BsmI* and *FokI* polymorphisms based on any of the BMD measurements.

In young adults (Study IV), the *FokI* polymorphism and the tertiles of urinary sodium excretion showed an interaction with urinary calcium excretion (sexes pooled, ANCOVA $p_{\text{interaction}} = 0.025$) (Figure 11, unpublished results). In females, the *FF* and *Ff* genotypes showed increased U-Ca due to increasing U-Na, but a similar association was not seen for the *ff* genotype (ANCOVA $p_{\text{interaction}} = 0.030$). However, in males, U-Ca also increased due to increasing U-Na in the *ff* genotype (ANCOVA $p_{\text{interaction}} = 0.081$). The interaction between U-Na and the *FokI* polymorphism did not have any effects on S-PTH levels or on any of the BMD measurements.

5.7 Gene-gene interactions (Study IV)

In young adults (Study IV), the interactions between the *BstBI* and *FokI* polymorphisms were significant for S-PTH (ANCOVA $p=0.041$, adjusted for sex, BMI, S-25OHD, S-iCa and S-P) and for U-Ca (ANCOVA $p=0.014$, adjusted for sex, BMI, S-25OHD, calcium intake and sodium excretion) (Publication IV, Figure 1). The *bbff* genotype had the highest S-PTH level (41.5 ng/l) and the lowest U-Ca (3.6 mmol/l). When sexes were analysed separately, the interactions between the *FokI* and *BstBI* polymorphisms and S-PTH and U-Ca were significant only in females (*BstBI* * *FokI* interaction for S-PTH $p=0.013$ and for U-Ca $p=0.046$). There were no significant interactions between the VDR, CaSR and PTH polymorphisms with any of the BMD measurements.

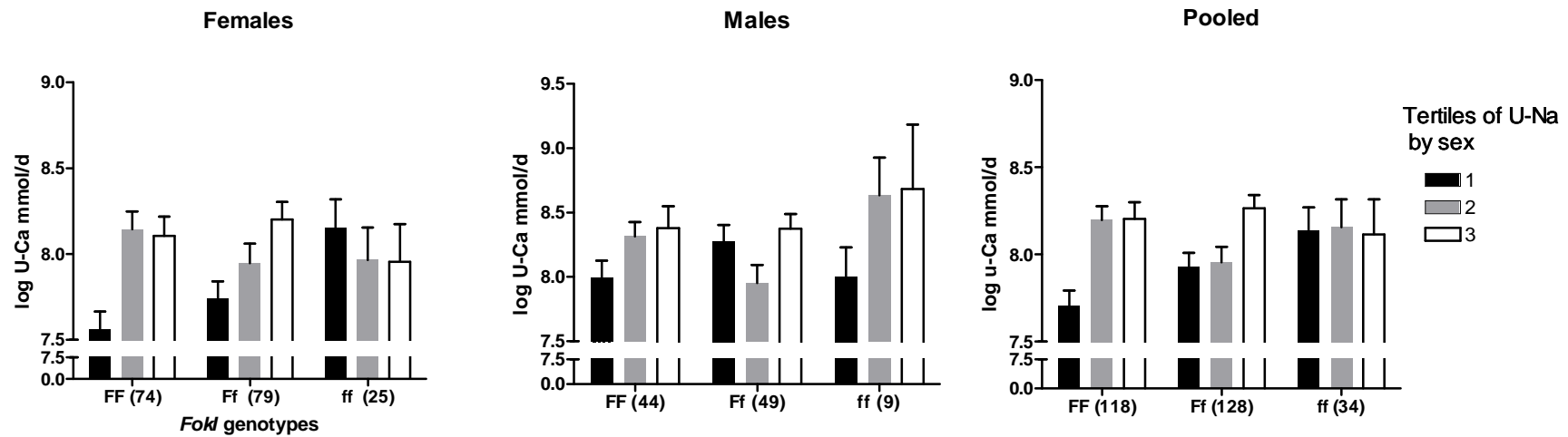


Figure 11 Urinary calcium excretion by *FokI* genotypes and tertiles of urinary sodium excretion. ANCOVA for urinary calcium excretion adjusted for BMI, calcium intake, S-25OHD and sex in the pooled group. ANCOVA p-values for the interaction between *FokI* genotypes and U-Na tertiles are 0.030, 0.081 and 0.025 for females, males and the pooled group, respectively.

6 DISCUSSION

During the past decade, significant progress has been made in revealing the genetic basis of bone health (Ferrari 2006, Liu et al. 2006, Ralston and de Combrugghe 2006). The number of candidate genes and SNPs linked to or associated with skeletal health has expanded and findings have been supported by multiple studies. On the other hand, the more associations identified, the more complex and inconclusive has the knowledge on skeletal genetics become. Furthermore, the expanded puzzle becomes even more complex after adjustment for confounding factors from the environment. Since the heritabilities of calcium homeostasis and bone strength are estimated to vary from 14% to 85%, the significant contribution of lifestyle and other non-genetic factors to bone health is indisputable. In spite of this, research on skeletal genetics has focused little effort on controlling for potential environmental confounders, which may partly explain discrepancies between the various findings so far (Weiss and Terwilliger 2000).

This thesis has a nutrigenetic approach to skeletal genetics: associations and interactions between the studied SNPs and nutritional factors with bone health have been evaluated. The findings of these studies are of great importance because evidence regarding the impact of genetics on skeletal health in the Finnish population is scarce. The main findings of this thesis were novel associations of the lactase C/T₋₁₃₉₁₀, the VDR *FokI*, the CaSR A986S and the PTH *BstBI* gene polymorphisms with calcium homeostasis and BMD. In addition, some results indicate that diet-gene and gene-gene interactions modulate the associations of the studied gene polymorphisms with calcium homeostasis.

6.1 Evaluation of the study designs and methods

It has been demonstrated that only 16% of genetic associations identified for complex traits have subsequently been replicated with formal statistical significance (Ioannidis et al. 2003a). The first study often suggests a stronger genetic effect that becomes gradually less prominent or even disappears as more data accumulate (Ioannidis et al. 2001). The inconsistent evidence for genotype-phenotype associations in the etiology of complex diseases may derive from several factors: lack of power due to small sample size, publication bias, confounding by genetic heterogeneity and population history, confounding by effect modification by other genetic or environmental factors, and limited or conflicting phenotypic characterization (Smith and Ebrahim 2003).

6.1.1 Sample size

The main cause of non-significant results in skeletal genetics is the lack of statistical power to detect effects of candidate gene SNPs that usually cause small phenotype differences within the normal physiological range. An adequately powered study to detect single genetic associations with complex traits requires at least one thousand subjects, since most associations have small odds ratios (1.1-1.5)(Ioannidis 2003b). However, due to a limited number of available DNA samples, most genetic association studies are small. The required sample size is even greater if the interactions between several gene variants or between environmental and genetic factors are investigated. Typically, larger studies suggest weaker associations or no associations at all compared with strong associations found in smaller studies. In addition, larger studies are costly and unlikely to be feasible for all research questions, such as environment-gene interactions. Genetic association studies require cautious replication; thus, although strong conclusions cannot be drawn from small studies, the findings provide valid data for meta-analyses (Ioannidis et al. 2001). Meta-analysis could help to separate the true associations from those that are false, and consortium approaches, in which individual subject data are contributed from several teams to common databases, should also be encouraged to overcome the lack of statistical power (Ioannidis 2003b). However, small studies with non-significant results are difficult to locate for meta-analysis and statistical approaches to correct missing studies are problematic (Duval and Tweedie 2000). It has been suggested that international registries where all researchers contribute their data along with international meta-analyses could diminish the threat of publication bias and provide more robust analyses of the biological significance of the SNPs (Ioannidis et al. 2002).

The studies in this thesis demonstrate both the strengths and weaknesses of small association studies. The major weakness is that, due to the small sample size, part of the the significant associations could have occurred by chance. The results section of this thesis includes 250 statistical tests and comparisons, of which 65 were significant at the < 0.05 probability level. The probability is that 12 of these significant results occurred by chance, indicating that 80% of the significant findings are statistically true associations. The main advantage of the small sample size is that it allowed inclusion of several indicators of calcium homeostasis and bone strength in the study design and allowed better control for confounding from the environment.

6.1.2 Genetic heterogeneity

It has been suggested that contradictory results in genetic association studies could derive from genetic heterogeneity of the study populations (Giguere and Rousseau 2000). Thus, isolated populations possess many advantages in the search for complex disease genes (Peltonen et al. 2000). Firstly, isolated populations show significantly less genetic diversity, because the populations start with a small group of founders and during the rapid expansion of the population they often experience bottlenecks, such as environmental disruption or infectious disease. Secondly, isolated populations have a higher level of linkage disequilibrium and reduced recombination (Shifman and Darvasi 2001). Isolated and outbred populations do not differ significantly in LD between SNPs at a shorter distance, but the greater the distance between SNPs, the higher the level of LD in the isolated population. Therefore, association studies in isolated populations have greater statistical power since the reduction in genetic heterogeneity increases genotypic relative risk and thus the ability to identify susceptibility genes for complex traits (Risch 2000). The great strength of the studies in this thesis is that the Finnish population has a low genetic diversity compared to other European populations due to an early settlement and subsequent rapid growth of the population without immigration from other gene pools (Kittles et al. 1998).

Human genetic variation is a result of genetic phenomena such as recombination and mutation rates and of evolutionary processes that include, for example, the history of the population size and structure, and natural selection (Przeworski et al. 2000, Zmuda et al. 2000, Rukin et al. 2007). Population history has genome-wide effects, while locus-specific forces such as natural selection shape only genetic variability at loci that are tightly linked to a locus under the pressure of natural selection. Therefore, deviations from expected allele or genotype distributions (Hardy-Weinberg equilibrium) across relevant phenotypic groups might help to identify functional candidate genes in disease etiology (Hoh et al. 2001). Alleles that are causative in disease etiology are expected to be overrepresented among cases with the disease and under-represented among disease-free individuals (Nielsen et al. 1998). However, departure from the Hardy-Weinberg equilibrium is one of the major confounding factors in association studies. Such deviations can bias the type I error rate such that the chance of a false positive association increases substantially if the proportion of the homozygotes with the high risk allele is more common in the general population than predicted by Hardy-Weinberg equilibrium (Schaid and Jacobsen 1999). Deviations from the expected genotype distribution could result from several factors such as genotyping errors, fluctuations due to small sample size, non-random mating migration into or out of the population, population stratification, and the admixture of different

ethnic groups (Zmuda et al. 2000). In Study III, the VDR genotype distribution deviated from the Hardy-Weinberg equilibrium, and was characterized by a higher than expected frequency of homozygotes. The explanation for this finding could be a selection bias because the subjects in Study III were volunteers rather than a randomly selected subgroup of the Finnish population. Therefore, the results from Study III should be interpreted with caution.

One limitation to the studies on the VDR gene polymorphisms is that haplotype analyses were not performed. Haplotype analyses are useful in genetic association studies because high levels of LD in a genomic area result in a limited number of haplotypes in that area; thus, a smaller number of polymorphisms have to be genotyped to "cover" the variance in a candidate gene (Thakkestian et al. 2004b, Uitterlinden et al. 2004). In addition, haplotype analyses in the candidate genes allow inclusion of the associations of the silent polymorphisms that are in LD with the causative SNP. The physical properties of proteins, such as folding kinetics and stability, may depend on the interactions between amino acid combinations, thus haplotypes may have a direct biological relevance (Rebeck et al. 2004). Most importantly, haplotype analyses and understanding of the LD patterns in candidate genes can explain the discrepant findings between varying populations or ethnic groups. Silent polymorphisms can serve as markers for truly functional loci elsewhere, but the LD within the marker haplotype and linkage with the functional alleles may differ between ethnic groups.

6.1.3 Phenotype heterogeneity

Genetic association studies are most successful when genetic markers can be examined in relation to a single intermediate phenotype that predicts disease outcome. However, gene polymorphisms may have pleiotrophic effects because they probably influence more than one intermediate phenotype (Smith and Ebrahim 2003). For example, vitamin D is involved in a number of endocrine systems including calcium homeostasis, immune modulation, and regulation of cell growth and differentiation, which all have a central role in the regulation of bone health. In addition, VDR gene polymorphisms have been shown to be associated with bone size (Fang et al. 2007) and muscle strength (Windelinckx et al. 2007), which both contribute to bone strength. If the VDR gene variant has opposite effects on calcium absorption efficiency and bone turnover, the net effect on BMD is different than expected from both effects individually. Nevertheless, examining the relationship of several determinants of calcium and bone metabolism with the SNP of interest may provide a more holistic view of the genetic association. This was evident in Study IV, where calcium homeostasis was examined by measuring the concentrations of calcium in the serum and urine in addition to PTH, which regulates these concentrations.

The study revealed a novel interaction between the VDR and PTH gene polymorphisms that was consistent both for urinary calcium excretion and PTH secretion.

Common chronic diseases have several intermediate endpoints, which increases the heterogeneity of findings from genetic association studies. For example, various determinants of bone strength are available, such as the measurement of bone mineral content and density by the DXA-method, bone geometry measurements provided by the pQCT-method, and QUS variables that are assumed to reflect the quality of bone structure. The situation is even more complex when investigating the final endpoint of osteoporosis, fractures, because a great number of the environmental and genetic factors contribute to bone fragility and fracture risk. Moreover, the varying penetrance of the investigated phenotype or genotype-related risks may obscure the gene-disease associations between populations. For instance, previous findings have shown that lactose tolerance may differ significantly between those with genetic lactose non-persistence (Enattah et al. 2005a, Gugatschka et al. 2005), and this thesis revealed that the consumption of low-lactose milk products diminishes differences in calcium intake between the lactase genotypes.

6.1.4 Environmental confounding

Several studies on osteoporosis candidate genes have ignored potential lifestyle-related confounders and effect modifiers such as calcium intake, physical activity, smoking and alcohol intake, which have a significant contribution to bone health. The great advantage of this study is that this methodological problem was overcome because lifestyle data were carefully recorded and included as confounders in the statistical analyses.

In contrast to the approach used in this thesis, Smith and Ebrahim (2003) have suggested that studies of the association between diseases and gene variants of known function may share with randomized controlled trials the advantage of excluding confounding as an explanation for apparent relations. According to this Mendelian randomization theory, in a population-based genotype-disease association study, the random assortment of alleles at the time of gamete formation (Mendel's second law) results in a random association between loci and disease in a population. One key point is that the distribution of such genotypes is unrelated to socioeconomic or behavioural confounders; thus, it is possible to exploit the random assignment of genes as a means of reducing confounding in examining exposure-disease associations. This strategy is most powerful when studying modifiable exposures that are poorly measured and considerably confounded, such as dietary factors. Nevertheless, the Mendelian randomization strategy has some caveats (Little and Khoury 2003) for which reasons the theory was not applied in the studies in this thesis. Firstly, the

distribution of some confounders differed significantly between the genotype groups, which may have a substantial effect on the results, especially in smaller studies. Secondly, Mendelian randomization does not take into account the possibility of varying patterns of LD between populations. Knowledge of the patterns of LD for some of the studied SNPs was lacking, which may explain the discordant results in comparison to results from other populations. Thirdly, the strategy proposed by Smith and Ebrahim (2003) depends on having an understanding of the gene function, which was limited for many of the studied SNPs in this thesis. Finally, one of the main aims of this thesis was to examine gene-nutrient interactions that are not included in the Mendelian randomization strategy.

A major methodological problem in studies examining diet-gene interactions is the misclassification of exposure, which attenuates the power to detect interactions and results in the need for larger sample sizes. Genetic variation affects nutrient digestion, absorption, metabolism, uptake and biotransformation; thus, several different types of biomarkers would be needed to assess the health consequences of the interactions between diet and genes (Milner 2000). Moreover, it is important to get time- and dose-dependent information on food components that modify the phenotype of interest. Because of the expense and limitations of detailed quantitative dietary assessment methods, the food frequency questionnaire (FFQ) is now the most commonly used dietary instrument in large epidemiologic studies (Tucker 2007). However, the major limitation of the FFQ method is that it poorly estimates the diet of individuals with divergent eating patterns, although they may be an interesting subgroup of the population when examining diet-gene interactions. Calibration of FFQs with dietary recall provides assurance that foods, recipes and portion sizes are correctly represented. The FFQs that were used in the present studies were validated with dietary recall method (Lamberg-Allardt et al. 2001). Furthermore, when possible, biomarkers with well-established validity, such as S-25OHD as a determinant of vitamin D status and urinary sodium excretion as a determinant of sodium intake, were assessed.

6.2 Associations of the studied SNPs with calcium homeostasis and BMD

6.2.1 The lactase gene C/T-₁₃₉₁₀ polymorphism

Wide evidence supports the essential role of regular milk consumption and adequate calcium intake in growing and maintaining a strong skeleton (Sandler et al. 1985, Soroko et al. 1994, Välimäki et al. 1994, Matkovic et al. 2004). It has been suggested that lactose intolerance could cause lower calcium intake due to avoidance of milk products, which causes predisposition to poorer bone health. Some studies indicate that if lactose intolerance is symptomatic and reduces calcium intake, it has detrimental effects on PBM or bone health at a later age (Honkanen et al. 1996, Honkanen et al. 1997, Goulding et al. 1999, DiStefano et al. 2002). However, the evidence is controversial and contradictory results have also been published (Slemenda et al. 1991, Kudlacek et al. 2002). Although genetic lactase non-persistence has been shown to correlate with decreased milk consumption and lower calcium intake by several studies (Obermayer-Pietsch et al. 2004, Gugatschka et al. 2005, Lember et al. 2006, Anthoni et al. 2007, Torniainen et al. 2007), so far only limited data indicate that this has harmful effects on bone health (Obermayer-Pietsch et al. 2004, Enattah et al. 2005b).

The lactase gene C/T-₁₃₉₁₀ polymorphism was associated with the type and amount of milk and milk products consumed but not with PBM

According to results from the Young Finns Study, the lower total calcium intake in the C/C-₁₃₉₁₀ genotype was mostly explained by lower milk consumption. In addition, the results indicate that preference for low-lactose milk and milk products may decrease the risk of inadequate calcium intake in those with the C/C-₁₃₉₁₀ genotype. Although in the Young Finns Study a significant proportion of those with the lactase non-persistence (C/C-₁₃₉₁₀) genotype had, from childhood, a calcium intake that was lower than recommended, the results do not support the hypothesis of lower PBM for those with genetic lactase non-persistence. This finding is in agreement with previous Finnish studies in young men and postmenopausal women that failed to support the relationship between genetic lactase non-persistence and poorer bone health (Enattah et al. 2004 and 2005a). When compared to Finnish studies, studies in other European populations have shown greater differences in calcium intake between lactase genotypes (Obermayer-Pietsch et al. 2004, Gugatschka et al. 2005, Lember et al. 2006). The habitual calcium intakes in adult Finns are higher

than in many other European countries (Paturi et al. 2008, European Commission 2003) due to the tradition of daily consumption of milk and milk products.

Previous studies indicate a poor correlation between molecularly confirmed lactase non-persistence and self-reported lactose intolerance (Enattah et al. 2005a, Gugatschka et al. 2005). In agreement with this, results from the Young Finns Study indicate that also many of those with genetic lactase persistence avoid milk drinking and follow low-lactose or milk-free diets. Several factors could explain the individual differences in lactose tolerance. Digestion of lactose depends not only on the dose of the lactose but also on the rate of gastric emptying and delivery of lactose to the colon (Leichter 1973, Martini and Savaiano 1988), and colonic bacterial adaptation to frequent lactose consumption (Johnson et al. 1993). Those with lactase non-persistence who have experienced gastrointestinal problems after consumption of large amounts of lactose may be psychologically sensitized to milk consumption (Suarez et al. 1997). Some findings suggest that females with lactose maldigestion experience stronger gastrointestinal symptoms than males (Vesa et al. 1998, Krause et al. 1996). In agreement with this finding, the Young Finns Study showed that low-lactose or milk-free diets were almost twice as common in females with the lactase non-persistence genotype than in males with the same genotype.

Because clinical symptoms and lactose malabsorption are poorly correlated and indirect diagnostic tests for lactose malabsorption are inaccurate, genetic testing for the lactase gene C/T₋₁₃₉₁₀ polymorphism could be used to improve diagnosis of lactase non-persistence (Rasinperä et al. 2004, Kerber et al. 2007, Schirru et al. 2007). For example, genetic testing could reduce the number of false negative results from the breath hydrogen test, be useful in determining lactase status of those individuals with borderline values in the breath hydrogen test and distinguish those with secondary causes of lactose maldigestion. However, there are several open questions before genetic testing for lactase non-persistence could be applied without misinterpretations. Firstly, although there is a consensus that lactase non-persistent individuals have lower levels of lactase mRNA, the differences in mRNA levels between the genotypes show a marked variation (Swallow 2003). This suggests that diet or other environmental factors can affect the lactase mRNA levels and cause considerable differences in lactose digestion irrespective of the genotype. Secondly, the down regulation of lactase activity is dependent on a child's age (Rasinperä et al. 2004), therefore it is not a valid test for smaller children. Besides, the causal polymorphism for lactase non-persistence differs between populations and the natural selection of lactase persistence at an adult age could even be explained by a selective event acting on a nearby gene (Poulter et al. 2003, Swallow et al. 2003). Furthermore, epigenetic mechanisms could modulate the penetrance of genetic lactase non-persistence due to the regular intake of lactose from milk products.

*Bone loss was more common in young adult males than in females, especially in the C/C.
13910 genotype*

The main result from the BMD follow-up in the Young Finns Study (Study I) is that bone loss in young adulthood was more common in males than in females. In both females and males, bone area continued to increase; however, in males increasing bone area was accompanied by decreasing BMC, resulting in a significant decrease in BMD. The sex-difference in bone loss is a highly valuable finding since current data on bone loss in young adulthood is contradictory (Krolner and Pors Nielsen 1982, Riggs et al. 1986, Buchanan et al. 1988, Rodin et al. 1990, Sowers et al. 1991, Mazess and Barden 1991, Recker et al. 1992, Ravn et al. 1994, Citron et al. 1995, Prior et al. 1996, Arlot et al. 1997, Sowers et al. 1998, Warming et al. 2002, Riggs et al. 2004, Uusi-Rasi et al. 2007, Riggs et al. 2008), and because very few studies have examined bone loss in young men (Aaron et al. 1987, Kalender et al. 1989, Riggs et al. 2004, Riggs et al. 2008). The youngest age cohort had the highest femoral neck BMC and BMD at the baseline (Välimäki et al. 1994) and the greatest bone loss at the femoral neck during the follow-up, whereas at the lumbar spine the baseline bone mass and changes during the follow-up did not differ between the age cohorts. This suggests that after PBM attainment, bone mass and density start to fall at the hip rather than at the lumbar spine, which comprises mainly of trabecular bone. This finding is discordant with prospective studies suggesting that bone loss in young adulthood occurs mainly at lumbar spine and trabecular bone sites (Krolner and Pors Nielsen 1982, Aaron et al. 1987, Buchanan et al. 1988, Kalender et al. 1989, Arlot et al. 1997, Riggs et al. 1986, Citron et al. 1995, Prior et al. 1996, Riggs et al. 2004, Riggs et al. 2008).

Another interesting finding from Study I was that in males, the C/C-13919 genotype seemed to be associated with an increased risk for greater bone loss in young adulthood. The explanation for these findings could depend on the changes in calcium intake and calcium homeostasis between childhood and adulthood. It may be that the lower calcium intake for those with C/C-13919 genotype is adequate for optimal PBM attainment, because adolescents have significantly higher net calcium absorption from a given calcium intake than adults (Weaver et al. 1995). In contrast, the period of young adulthood is characterized by a decline in calcium absorption and calcium retention with a concomitant increase in calcium output (Matkovic and Heaney 1992, Weaver et al. 1995, Wastney et al. 1996). Matkovic and Heaney (1992) have suggested that in order to offset the higher urinary calcium output and to provide enough calcium for bone building from 19 up to 30 years, calcium intake should be above the threshold of 960 mg/d. Thus, when calcium intake decreased in males in young adulthood, the amount of calcium may have been insufficient for maintaining bone mass. Although the decrease in calcium intake during the follow-up in young adulthood did not differ between the lactase genotypes in either sex, in

one third of females and in one fourth of males with the C/C₋₁₃₉₁₀ genotype, daily calcium intake did not meet the Finnish Nutritional Recommendations (2005). Moreover, calcium intake predicted changes in bone mass more than the lactase genotype. This could be due to good availability and common use of low-lactose and lactose-free products in Finland, which may protect those with the C/C₋₁₃₉₁₀ genotype from inadequate calcium intake and deterioration of bone health.

The main weakness of Study I is the small sample size, thus, our findings should be confirmed by large studies. Other limitations of the study arise from the DXA method, which may cause misinterpretation of the results from the follow-up study (Lenchik et al. 2002). For a method to be well suited to longitudinal BMD monitoring, it would need to have a small precision error compared to the expected BMD change. Regarding precision error due to the operator and positioning of the patient, an advantage of the 12-year follow-up study was that the DXA operators were well educated and had long experience with bone density measurement. Furthermore, the instability of the scanners during the follow-up may cause poorer precision (Hangartner 2007), but this was not a problem in this study since there were only small drifts in the lumbar spine phantom measurements between the baseline and follow-up. Changes in the ratio of extraosseous fat and lean muscle tissue within the DXA scan region can produce inaccuracies in repeated BMD measurements (Kuiper et al. 1996, Bolotin et al. 2003). Unfortunately, changes in body composition during the follow-up were not assessed; however, the observed weight changes do suggest that the subjects gained fat mass during the follow-up years. The increased amount of fat tissue causes underestimation of bone loss, which further confirms the findings of bone loss in Study I. For more specific information on tissue-specific changes in bone geometry and strength, the pQCT method should have been used. The DXA method does not have the capacity to distinguish between cortical and trabecular bone tissue or to accurately scan the age-related enlargement of bones that results from endocortical bone resorption accompanied by periosteal bone formation (Seeman 2001, Duan et al. 2003).

6.2.2 The VDR gene *BsmI* and *FokI* polymorphisms

The possible role of VDR gene variation in osteoporosis susceptibility has been a subject of intense investigation for several years. Numerous studies have investigated the effects of VDR gene polymorphisms, individually or in interaction with diet and other genetic factors, on several biological endpoints including calcium absorption, BMD, bone loss and fractures. In spite of this, the effects of individual VDR gene polymorphisms have been very modest and the results from different studies are not all in agreement. The earliest meta-analyses supported an association between the *BsmI* polymorphism or the 3'UTR

haplotypes and BMD (Cooper and Umbach 1996, Gong et al. 1999a, Thakkestian et al. 2004a, Thakkestian et al. 2004b), whereas the more recent meta-analyses and large scale studies on the 3'UTR haplotypes and functional VDR polymorphisms on bone health suggest a lack of association (Fang et al. 2006, MacDonald et al. 2006, Uitterlinden et al. 2006) or have found a relationship only with fractures (Grundberg et al. 2007, Moffett et al. 2007).

The VDR gene BsmI and FokI polymorphisms were associated with BMD

The results from Study III showed no association of the VDR *BsmI* polymorphism with calcium absorption efficiency, nor with markers of bone turnover in pre- and postmenopausal women. Nevertheless, Study III indicated a trend toward higher lumbar spine BMD for the *BB* genotype, and the results from Study II in adolescents supported the hypotheses of an association between the *B* allele and higher BMD. These findings do not agree with the results from a meta-analysis by Cooper and Umbach (1996) and from the Young Finns Study (Viitanen et al. 1996), which both showed an association between the *bb* genotype and higher BMD. The meta-analysis by Cooper and Umbach (1996) suggested that age and stage of skeletal lifespan contribute to the association between *BsmI* genotypes and BMD, thus explaining contradictory findings from different studies. Moreover, interactions with lifestyle factors could modulate the association between the *BsmI* genotype and BMD. For instance, the discordant results could be due to differences in calcium absorption efficiency with low calcium intake between the *BsmI* genotypes (Dawson-Hughes et al. 1995, Gennari et al. 1997). Besides, the different ethnic backgrounds of the subjects could also explain inconsistent findings in the relationship between *BsmI* polymorphisms and bone health (Zmuda et al. 1997).

Study II in adolescents showed that the *Ff* genotype was associated with higher BMD as measured with QUS; yet previous studies have shown lower BMD for the *f* allele in other populations (Gross et al. 1996, Arai et al. 1997, Gennari et al. 1999). However, Study IV in young adults did not reveal any associations between the *FokI* polymorphism and the determinants of calcium homeostasis and peripheral BMD (DXA method) in young adults. This result agrees with some previous studies that indicate a lack of association between the *FokI* polymorphism and BMD (Eccleshall et al. 1998, Ferrari et al. 1998). The discrepancies between the results of the studies could be explained by differences in the site and composition of the bone measured. Study II suggested a stronger association of the *FokI* polymorphism with the QUS measurements, especially with BUA, rather than with the distal forearm DXA measurements. Distal radius and ulna sites consist more of cortical bone than calcaneus, which is mainly trabecular bone (Baron 1993). Trabecular bone is metabolically more active than cortical bone and could therefore be more

responsive to genetically modulated effects of lifestyle on bone strength (Dequecker et al. 1987, Pocock et al. 1987). It has been suggested that the measured total ultrasound attenuation reflects some aspects of trabecular architecture in addition to BMD (Kaczmarek et al. 2000, Nicholson et al. 2001, Cortet et al. 2004). Thus, the results may indicate that the *FokI* polymorphism could contribute more to either the structural properties of bone strength or the collagen composition rather than to BMD.

An interpretation of the relationships between the *BsmI* polymorphism and bone health is difficult due to the non-functional nature of the polymorphism; however, there are several hypotheses suggesting a possible mechanism underlying the effects of this VDR gene polymorphism. The *BsmI* polymorphism is located in the same haplotype block with the polymorphisms of the 3'UTR region (Morrison et al. 1992, Ingles et al. 1997, Durrin et al. 1999). Because the 3'UTR region regulates the stability of the messenger RNA (mRNA), SNPs in the 3'UTR region could be responsible for differences in the availability of mRNA for translation into the receptor protein. For instance, two large-scale studies have demonstrated that VDR 3'UTR haplotypes were associated with VDR mRNA levels, which might contribute to the higher fracture incidence for the risk haplotype (Fang et al. 2005, Grundberg et al. 2007). The mechanism underlying the bone mass differences between the *FokI* genotypes relies on the finding that the shorter receptor protein (*F* allele) may play a more active role in VDR-responsive gene expression because it interacts more efficiently with transcriptional factor IIB than the full-length receptor protein (*f* allele) (Arai et al. 1997, Jurutka et al. 2000). In contrast, some evidence shows no functional differences between the alleles (Gross et al. 1998).

One limitation of the Study II was that the stage of puberty was not assessed, although it has been suggested that the DXA values at the radius are not affected by puberty (Zanchetta et al. 1995). The findings of an association between the *FokI* polymorphism and BMD in adolescents but not in adults suggests that the contribution of the genetic factors to bone strength is most distinct at a young age, slowly diminishing later in life due to the long exposure to lifestyle factors. Furthermore, the influence of the genetic component of BMD may differ between sexes, as was seen in Study II.

6.2.3 The CaSR gene A986S polymorphism

In previous studies, the 986S allele of the CaSR gene has been shown to be associated with higher than average serum total calcium levels (Cole et al. 1999, Cole et al. 2001, Lorenzon et al. 2001, Eckstein et al. 2002) and ionized calcium levels (Scillitani et al. 2004). However, the association between A986S polymorphism and BMD is contradictory (Lorenzon et al. 2001, Eckstein et al. 2002, Takacs et al. 2003, Cetani et al. 2003, Bollerslev et al. 2004, Perez-Castrillón et al. 2006).

CaSR gene A986S polymorphism was related to serum ionized calcium concentration

The results from Study IV are in congruence with the previous studies showing higher S-iCa concentrations for the combined group of AS and SS genotypes than for the AA-genotype of the CaSR A986S polymorphism (Scillitani et al. 2004). However, results from several studies have failed to show this association or have been contradictory (Miedlich et al. 2001, Cetani et al. 2002, Young et al. 2003, Bollerslev et al. 2004, Perez-Castillion et al. 2006). Although the S allele was found to be associated with higher S-iCa levels in Study IV, there were no differences in S-PTH or in urinary calcium excretion between the A986S genotypes and no indications of a direct effect of the A986S polymorphism on BMD. The inconsistent findings could be due to large ethnic differences in the allele frequencies of the three SNPs that are in LD, A986S, R990G and Q1011E, making it difficult to isolate the effects of a single SNP (Yun et al. 2007). Thus, the CaSR haplotype may be a better predictor of calcium homeostasis than any single genotype alone (Scillitani et al. 2004).

The CaSR gene polymorphisms could alter the activity of the receptor protein, leading to differences in calcium homeostasis. For example, the decreased receptor activity may increase the set point of CaSR sensitivity for ionized calcium in the kidneys or parathyroid gland, resulting in an elevated serum calcium concentration. Regarding the functional differences in CaSR between the A986S genotypes, the S-allele is suggested to be the “hypercalcemic” or “Ca²⁺ resistant” variant that needs higher serum ionized calcium concentrations for suppression of PTH secretion and for an increase in urinary calcium excretion (Cole et al. 2001). Caucasians have a higher prevalence of the S allele than, for example, the Japanese (Kanzawa et al. 1999). This could result from the long history of low vitamin D intake among Europeans at higher latitudes, causing positive natural selection in favour of those with the “calcium-conserving” S-allele (Cole et al. 2001).

The differences in CaSR activity in the different A986S genotypes could be due to amino-acid substitutions in the receptor protein. The amino-acid substitutions that result from the A986S polymorphism may change the local electric charge distribution on the intracellular domain of CaSR, causing a change in the spatial conformation and folding of the protein (Smith et al. 1996). However, results from a functional characterization study indicate that the CaSR polymorphisms at codons 986, 990 and 1011 do not lead to alterations in the set points of CaSR activity (Harding et al. 2006). Functional vitamin D responsive elements have been identified in the promoter region of CaSR, and CaSR gene transcription is activated by 1,25(OH)₂D (Canaff and Hendy 2002). Since vitamin D status is a potential confounder in the relationship between the A986S polymorphism and calcium homeostasis, the analyses in Study IV were performed by adjusting for the S-25OHD concentrations.

6.2.4 The PTH gene *BstBI* polymorphism

Previous studies suggested that the PTH *BstBI* polymorphism is associated with BMD or bone dimensions in populations of Japanese (Hosoi et al. 1999, Kanzawa et al. 1999, Katsumata et al. 2002), Chinese (Dvornyk et al. 2005) and Caucasian (Gong et al. 1999b, Deng et al. 2002) origin. The mechanisms by which the PTH gene polymorphisms could contribute to differences in calcium and bone metabolism consist of modification of PTH secretion, degradation or action. However, evidence for variation in calcium homeostasis due to the *BstBI* gene polymorphism is minimal since only a few studies indicate a relationship between the *BstBI* polymorphism and serum calcium concentration and markers for bone turnover (Hosoi et al. 1999, Kanzawa et al. 1999).

The PTH gene BstBI polymorphism contributed to BMD

The results from Study IV are in agreement with previous results suggesting better bone health for those with the *B* allele (Hosoi et al. 1999, Kanzawa et al. 1999, Katsumata et al. 2002, Gong et al. 1999b). In contrast, some results indicate a lack of association between the *BstBI* polymorphism and BMD (Zhou et al. 2003, Lei et al. 2005). In agreement with the findings of Goswami et al. (2004), the results from Study IV indicated no differences in the markers for calcium metabolism between the *BstBI* genotypes. This finding is discordant with the results from Japanese studies (Hosoi et al. 1999, Kanzawa et al. 1999), which could be due to confounding factors that are involved in the regulation of PTH gene expression. It has been proposed that serum calcium and phosphate concentrations determine PTH mRNA stability through the balance between stabilizing and degrading factors that interact with the mRNA (Naveh-Mani and Nechemia 2007). Hypocalcemia prevents PTH mRNA degradation, resulting in higher levels of PTH mRNA, and

hypophosphatemia leads to PTH mRNA decay and decreased PTH mRNA levels. In Study IV, the PTH concentrations were adjusted for serum calcium and phosphate concentrations. The Japanese studies examining the relationship between the *BstBI* polymorphism and PTH levels have not taken these confounders into consideration (Hosoi et al. 1999, Kanzawa et al. 1999).

The *BstBI* polymorphism is located in the non-coding region of the PTH gene, yet the locus is 50 nucleotides upstream of the 3' splice acceptor site. Goswami et al. (2004) have introduced an interesting hypothesis suggesting that the *BstBI* polymorphism could alter the splicing of the PTH-pre-mRNA. In the process of splicing, a short sequence of the intronic region, termed a branch point consensus nucleotide sequence, functions as the recognition signal for the spliceosome, which then excises the intron in the form of a lariat (Shapiro and Senapathy 1987, Senapathy et al. 1990). This regulatory sequence is characterized by a highly conserved adenosine residue, which serves as an anchor for the formation of the spliceosome. Nevertheless, further analysis showed that a role for the *BstBI* gene polymorphism in regulation of splicing of PTH-pre-mRNA was unlikely since a perfect branch point sequence existed 30 nucleotides upstream of the 3' splice acceptor. Another possible explanation for the associations of the *BstBI* polymorphism with PTH is linkage with another SNP in the PTH gene or in a nearby gene. The *BstBI* site is located in the region that also includes genes for calcitonin and insulin-like growth factor II (IGF II). Furthermore, a mutation in the first nucleotide of intron 2 has been reported to cause exon skipping in patients suffering from autosomal recessive isolated hypoparathyroidism (Parkinson and Thakker 1992), yet there was no linkage between the mutation and the *BstBI* polymorphism (Parkinson et al. 1993). As long as the biological meaning of this intronic polymorphism remains unresolved, the *BstBI* polymorphism should be considered as a marker of the chromosomal region adjacent to the PTH gene that is associated with variation in BMD.

6.2.5 Summary of the main findings of the studied SNPs

Table 11 summarizes the main findings from Studies I-IV, and compares the results to previous evidence.

Table 11. Summary of the main findings of the studied SNPs.

Studied SNPs	Main findings from this study	Findings from other studies
Lactase gene C/T₋₁₃₉₁₀ polymorphism (Study I)	<ol style="list-style-type: none"> 1. The C/C₋₁₃₉₁₀ genotype was associated with lower consumption of milk from childhood, predisposing females in particular to inadequate calcium intake. Consumption of low-lactose milk products decreased the risk of inadequate calcium intake. 2. Bone loss was more common in young adult males than in females, especially in the C/C₋₁₃₉₁₀ genotype; however, calcium intake predicts changes in bone mass more than lactase genotype. 	<ul style="list-style-type: none"> - The C/C₋₁₃₉₁₀ genotype was associated with decreased milk consumption and low calcium intake in 5 studies (Obermayer-Pietsch et al. 2004, Gugatschka et al. 2005, Lember et al. 2006, Anthoni et al. 2007, Torniainen et al. 2007). - The C/C₋₁₃₉₁₀ genotype was associated with deteriorated bone health in 2 studies (Obermayer-Pietsch et al. 2004, Enattah et al. 2005b).
VDR gene BsmI and FokI polymorphisms (Studies II-IV)	<ol style="list-style-type: none"> 1. The Bb genotype was associated with the lowest BMC values at the distal forearm and the Ff genotype with the highest calcaneal BUA values in adolescents (Study II). 2. No association was found between the VDR BsmI polymorphism and calcium absorption or markers for bone remodelling in pre- and postmenopausal women (Study III). 3. For the BB genotype, an association with higher S-OHD and a trend toward higher lumbar spine BMD was found (Study III). 4. No association was found between the FokI polymorphism and calcium homeostasis or BMD in young adults (Study IV). 	<ul style="list-style-type: none"> - An association between the BsmI polymorphism and BMD was found in 4 meta-analyses (Cooper and Umbach 1996, Gong et al. 1999a, Thakkestian et al. 2004a, Thakkestian et al. 2004b) and a lack of association with BMD in 3 meta-analyses (Fang et al. 2006, MacDonald et al. 2006, Uitterlinden et al. 2006). - The bb genotype was associated with better bone health (Dawson-Hughes et al. 1995, Cooper and Umbach 1996, Viitanen et al. 1996, Gennari et al. 1997, Zmuda et al. 1997). - The f allele was associated with lower BMD in 3 studies (Gross et al. 1996, Arai et al. 1997, Gennari et al. 1999), and a lack of association between FokI genotypes and BMD was found in 2 studies (Eccleshall et al. 1998, Ferrari et al. 1998).
CaSR gene A986S polymorphism (Study IV)	<ol style="list-style-type: none"> 1. S-iCa concentrations were higher for the AS and SS genotypes than for the AA genotype. 2. No association was found between A986S genotypes and other markers of calcium homeostasis, forearm BMD or calcaneal QUS values. 	<ul style="list-style-type: none"> - An association with S-Ca or S-iCa was found in 5 studies (Cole et al. 1999, Cole et al. 2001, Lorenzon et al. 2001, Eckstein et al. 2002, Scillitani et al. 2004). - Results were contradictory or no association with S-iCa was found in 5 studies (Young et al. 2003, Miedlich et al. 2001, Cetani et al. 2002, Bollerslev et al. 2004, Perez-Castrillón et al. 2006).
PTH gene BstBI polymorphism (Study IV)	<ol style="list-style-type: none"> 1. Forearm BMD and calcaneal QUS values were lowest for the bb genotype, but no association was found with calcium homeostasis. 2. An interaction was found between FokI genotypes and sodium intake on urinary calcium excretion. 3. An interaction was found between FokI and BstBI polymorphisms on PTH secretion and urinary calcium excretion. 	<ul style="list-style-type: none"> - An association with BMD or bone dimensions was found in 6 studies (Hosoi et al. 1999, Kanzawa et al. 1999, Katsumata et al. 2002, Gong et al. 1999b, Deng et al. 2002, Dvornyk et al. 2005).

6.3 Diet-gene and gene-gene interactions

The only significant diet-gene interaction that was revealed by the research described in this thesis, was the interaction effect between the *FokI* polymorphism and sodium intake on urinary calcium excretion (Study IV, unpublished results). This effect was seen especially in females, and in the *F* allele U-Ca increased when sodium intake (measured as U-Na) increased. This interaction still existed after adjusting for calcium intake and vitamin D status. In a trial with a calciuric diet, Harrington et al. (2004) also found an interaction effect between high sodium intake and the *FokI* genotype on calcium and bone metabolism. According to their results the *FokI* genotypes showed no differences in urinary calcium excretion when adapting to a high-sodium and high-protein diet, but this calciuric diet significantly increased urinary NTx, a marker of bone resorption. They concluded that in those with the functionally less active *f* allele, intestinal calcium absorption and renal calcium reabsorption did not compensate for calcium losses induced by a calciuric diet but instead the losses were compensated for increased bone resorption. This explanation would otherwise fit the results from Study IV, except that no differences were observed in the S-PTH concentrations or the BMD measurements and, unfortunately, the bone remodelling markers were not measured.

In Study IV, novel interaction effects of the VDR and PTH gene polymorphisms on the S-PTH and U-Ca concentrations were observed. The transcriptionally more active VDR *F* allele has been shown to be associated with lower S-PTH levels (Zofkova et al. 2003). Interestingly, our results showed that the *ff* genotype was associated with increased S-PTH only in the presence of the *bb* genotype. In addition, U-Ca was lower in the *bbff* genotype than in the other genotypes, which further supports the findings on PTH concentrations. The coordinated activities of PTH and 1,25(OH)₂D in maintaining serum calcium concentrations provide potential biological mechanisms to explain these gene-gene interactions. It has been shown that VDR mediates the suppressive effect of 1,25(OH)₂D on PTH secretion (Demay et al. 1992), and that the VDR polymorphism is associated with PTH secretion (Carling et al. 1995 and 1997, Yokoyama et al. 1998). Moreover, 1,25(OH)₂D and PTH act in concert to increase renal reabsorption of calcium (DeLuca and Zierold 1998). It has also been demonstrated that PTH lowers renal vitamin D receptor expression *in vivo* (Healy et al. 2005).

6.4 Significance of the nutrigenetic studies

Understanding the interrelationships among genes, gene products, and dietary habits is fundamental for identifying those who will benefit most from or be placed at risk by dietary intervention strategies (Joost et al. 2007). Genotyping offers the prospect of starting early in the prevention of disease, which is particularly important for diseases with a long period of development and irreversible pathology, such as osteoporosis. In theory, the availability of genomic data raises the possibility of personalizing the diet for optimal health and disease prevention. Targeting advice to those who would be most likely to benefit could save resources and increase the motivation of individuals at high risk to comply with dietary recommendations. On the other hand, in some individuals the response to dietary intervention might be poor due to their heredity. Thus, nutrigenetic information could help to identify those for whom changes in dietary habits will not be helpful and who will require medical treatment for disease prevention.

Nutrigenetics aims to identify the genetic variations that account for individual requirements for nutrients in much the same way as pharmacogenetics aims to identify the polymorphisms that affect drug efficacy and safety; however, the impact of genetic variation on nutritional requirements is more subtle (Stover 2006). A long exposure to a certain dietary pattern may be necessary for disease development, whereas the impact of pharmaceutical agents occurs mostly over a shorter period. Moreover, medications are prescribed in exact amounts but the recommended intake of a nutrient is a window of intake between a sufficient amount for 97% of healthy individuals and the highest safe intake that does not have any harmful effect for most individuals. With personalized nutrient recommendations, these parameters could be adjusted to accommodate the genetic variation found in the utilization of nutrients between individuals. Furthermore, genetics is driving a re-evaluation of nutritional inadequacy and safe upper limits for intake because advances in genomics will help in the search for new biomarkers that can define nutritional needs more precisely. The practical applications of nutrigenetics could be important for the food industry, raising the possibility of developing novel foods that are more nutritious and health-promoting than the products manufactured today (Kaput 2007). Low-lactose and lactose-free products are examples of novel foods that may prevent nutritional inadequacies that are produced by heredity. However, in view of the current evidence, more research is needed before genetic tests of lactase non-persistence can be applied in practice and used as a guide for dietary counselling.

Currently, there are already several commercial enterprises that provide genome-based preventive health care. However, there are many uncertainties and ethical and social

implications that should be evaluated before extending genetic testing beyond the counselling-intensive, high penetrance disorders (Hunter 2005). Affordable individual genome sequencing will be the easy part, but developing a credible database on replicable gene-environment interactions will be the big challenge. The main risk related to genetic testing is that recommendations and decisions may be based on insufficient or even misleading data that overwhelms the phenotypic medical data (Joost et al. 2007). For example, gene-gene interactions, epigenetics and low penetrance of disease alleles increase the number of puzzles and make the picture of personalized nutrition very diversified. Because social, economic and cultural factors are critical in the development of individual dietary patterns, they should also be included when designing studies for the identification of diet-gene interactions (Kaput et al. 2005).

Inherited susceptibility as a cause of disease could support a concept of genetic determinism that may have adverse effects on public health policy and could be misused to create inequity in health promotion of individuals (Slamet-Loedin and Jenie 2007). The true risk is the misuse of personal genetic data by 'interested third parties' such as employers and insurance companies (Joost et al. 2007). The concept of 'personalized prevention' also conflicts with the view that population-wide interventions are usually more effective in reducing incidence of common diseases than interventions that target high-risk individuals (Rose 1985). Therefore, even at this early stage of the development of nutritional genomics, efforts are needed to improve public awareness, public participation and consultation and to create collaborations between researchers, public policy makers and health care professionals.

7 CONCLUSIONS AND FUTURE PROSPECTS

- 1) Genetic lactase non-persistence (the C/C₋₁₃₉₁₀ genotype) was associated with lower consumption of milk from childhood, predisposing females in particular to inadequate calcium intake. In those with the C/C₋₁₃₉₁₀ genotype, low-lactose or milk-free diets were more common than in the T/T or C/T₋₁₃₉₁₀ genotypes, and a consumption of low-lactose milk and milk products decreased the risk for inadequate calcium intake. Males were more susceptible to bone loss after the years of peak bone mass attainment, and especially those with the C/C₋₁₃₉₁₀ genotype seemed to be at greater risk. However, calcium intake predicted changes in bone mass more than the lactase genotype. Thus, the results suggest that in Finnish population, a preference for low-lactose milk and milk products may protect those with the C/C₋₁₃₉₁₀ genotype from deterioration of bone health.
- 2) In adolescents, the *BsmI* polymorphism of the vitamin D receptor (VDR) gene was associated with BMC at the distal forearm and the *FokI* polymorphism with BMD at the calcaneus. In adults, the *FokI* polymorphism was not related to determinants of calcium and bone metabolism or to peripheral BMD. The *BsmI* polymorphism was not associated with intestinal calcium absorption in pre- and postmenopausal women but showed an association with vitamin D status and a trend toward an association with lumbar spine BMD. These findings suggest that the contribution of the *BsmI* polymorphism to bone health might depend on age, yet the study populations were too small to make a strong conclusion.
- 3) The 986S allele of the calcium sensing receptor (CaSR) gene was associated with higher serum ionized calcium concentrations, which confirms the results from some previous studies in other populations. BMD at the distal forearm and ultrasound attenuation (BUA) at the calcaneus were associated with the PTH *BstBI* polymorphism, but because no differences were found in the markers for calcium and bone metabolism, the mechanism by which this polymorphism regulates BMD requires further study.
- 4) The *FokI* polymorphism and sodium intake showed an interaction effect on urinary calcium excretion.
- 5) A novel gene-gene interaction was found between the VDR *FokI* and PTH *BstBI* gene polymorphisms in the regulation of PTH secretion and urinary calcium excretion.

These findings allow formulation of a few hypotheses for further studies. Since the results were inconsistent between subjects of varying ages, the contributions of the studied SNPs to calcium homeostasis and bone health should be evaluated in larger studies with subjects at varying stages of the skeletal life span. Also subjects at the opposite tails of the variance in calcium homeostasis and bone strength should be studied. Furthermore, more detailed determinations of the bone phenotypes, including measurements of bone geometry and separation of the trabecular and cortical bone tissues, are needed in order to get a more holistic view of the associations between candidate gene polymorphisms and bone strength. Finally, along with the genome-wide search for osteoporosis susceptibility genes, the diet-gene and gene-gene interactions in the associations between SNPs and calcium homeostasis and bone phenotypes should be examined in large population-based and carefully designed studies. The funding of the nutrigenetic research should be increased since the study designs require large sample sizes combined with expensive analysing methods and highly trained personnel.

Nutritional genomics may provide several benefits to human health. Increasing evidence for diet-gene interactions raises the possibility of identifying specific nutrient needs in different populations and redefining the nutrient recommendations to match more precisely the genetic characteristics of the population. Moreover, it helps in developing new diagnostic tests for adverse responses to diets and in improving the methodology for dietary assessment. Finally, nutritional genomics may provide information for planning personalized dietary patterns that are more nutritious, promote health and prevent, mitigate or cure disease. However, evidence-based population-wide interventions should concentrate on the well-known genetic risks of chronic diseases that are highly prevalent. Furthermore, because in the near future there will not be enough evidence for a fully holistic view of diet-gene interactions, it will be more efficient to target resources in disease prevention to high-risk populations or to high-risk subgroups at the population level rather than promote personalized nutrition.

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